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<b>(21) International Application Number:</b> PCT/US96/09627 <b>(22) International Filing Date:</b> 7 June 1996 (07.06.96)  <b>(30) Priority Data:</b> 08/485,340 7 June 1995 (07.06.95) US  <b>(71) Applicant:</b> RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; Suite 600, 101 N. Wilmot Road, Tucson, AZ 85711-3335 (US).  <b>(72) Inventor:</b> SELAWRY, Helena, P.; 7041 Woodlake Drive, Memphis, TN 38119 (US).  <b>(74) Agents:</b> DiGIGLIO, Frank, S. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).		<b>(81) Designated States:</b> AU, CA, JP, MX, NO, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> USE OF CO-LOCALIZED ISLETS AND SERTOLI CELLS IN XENOGRAFT CELLULAR TRANSPLANTS  <b>(57) Abstract</b>  The present invention describes a method of treating a disease that results from a deficiency of a biological factor which comprises administering to a mammal Sertoli cells and cells that produce the biological factor. In particular, the present invention describes a method of treating diabetes mellitus by transplanting pancreatic islet of Langerhans cells in conjunction with Sertoli cells to create an immunologically privileged site. A method of creating an immunologically privileged site and providing cell stimulatory factors in a mammal for transplants is further described by the present invention. A method of co-localizing islet cells with Sertoli cells and the use of the co-localized product in treating diabetes mellitus is further provided. The present invention further describes a method of creating systemic tolerance to foreign antigens. A method of enhancing the viability, maturation, proliferation of functional capacity of cells in tissue culture is further provided. A pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor is also provided.		

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USE OF CO-LOCALIZED ISLETS AND  
SERTOLI CELLS IN XENOGRAFT CELLULAR TRANSPLANTS

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5                   This invention was made with United States  
government support under grant DK42421 awarded by the  
National Institutes of Health. The United States  
Government may have certain rights in the invention.

CROSS-REFERENCE OF RELATED APPLICATIONS

10                   This application is a continuation-in-part  
of U.S. Serial No. 08/485,340 filed on June 7, 1995  
which is a continuation-in-part of U.S. Serial No.  
08/421,641 filed on April 13, 1995 which is a  
continuation-in-part of U.S. Serial No. 08/211,695  
filed on April 13, 1994.

15

FIELD OF THE INVENTION

                  Transplants of healthy organs or cells into  
a patient suffering from a disease are often rejected  
by the body due to an immune response initiated in  
20                   response to the foreign tissue or cells. The present  
invention provides a method of cellular  
transplantation in which an immunologically privileged  
site is created and cell stimulatory factors are  
produced, thus alleviating the rejection associated  
25                   with conventional transplantation therapy.  
Specifically, the present invention describes a method  
of treating a disease that results from a deficiency  
of a biological factor which comprises administering  
to a mammal Sertoli cells and cells that produce the  
30                   biological factor. In particular, the present  
invention describes a method of treating diabetes

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1    mellitus by transplanting pancreatic islet of  
Langerhans cells in conjunction with Sertoli cells to  
create an immunologically privileged site and to  
provide pancreatic islet cell stimulatory factors. A  
5    method of creating an immunologically privileged site  
and providing cell stimulatory factors in a mammal for  
transplants is further described by the present  
invention. A method of creating systemic tolerance to  
transplants is further provided by the present  
10    invention. The present invention further describes a  
method of enhancing the maturation, proliferation and  
functional capacity of cells in tissue culture by co-  
culturing these cells with Sertoli cells. A method of  
enhancing the recovery rate and viability of frozen  
15    cells, and in particular factor producing cells, in  
tissue culture by co-culturing these cells with  
Sertoli cells is also described herein. Another  
aspect of the present invention is directed to a  
method of co-localizing Sertoli cells with cells that  
20    produce a biological factor for treating diseases  
caused by a deficiency thereof, e.g., encapsulating  
islet cells which produce insulin with Sertoli cells.  
The use of the co-localized, e.g., encapsulated  
Sertoli cells and islet cells for treating diabetes  
25    mellitus is further described by the present  
invention. A pharmaceutical composition comprising  
Sertoli cells and cells that produce a biological  
factor is also provided.

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35

1    BACKGROUND OF THE INVENTION

          Certain chronic diseases destroy the  
functional cells in affected organs. Mammals with  
such diseases are often unable to produce proteins or  
5 hormones necessary to maintain homeostasis and usually  
require numerous exogenous substances to survive.  
Transplanting healthy organs or cells into a mammal  
suffering from such a disease may be necessary to save  
the mammal's life. This type of therapy is generally  
10 regarded as a last alternative to curing an otherwise  
fatal condition. Such transplants, however, are often  
rejected by the body due to an immune response  
initiated in response to the foreign tissue or cells.  
Presently, the only recourse to combat this immune  
15 response is to administer chronic nonspecific  
immunosuppression agents. Unfortunately, this only  
trades the complications of one chronic disease with  
other complications caused by the immunosuppression  
agent.

20           One disease which scientists have attempted  
to treat with organ and/or cellular transplants but  
have had very limited success is diabetes mellitus.  
Diabetes mellitus is a prevalent degenerative disease  
in mammals. It is characterized by a relative or  
25 complete lack of insulin secretion by the beta cells  
within the islets of Langerhans of the pancreas or by  
defective insulin receptors.

          This insulin deficiency prevents normal  
regulation of blood glucose levels and often leads to  
30 hyperglycemia and ketoacidosis. When administered to  
a mammal, insulin promotes glucose utilization,

- 1 protein synthesis, formation and storage of neutral lipids and the growth of certain cell types.

In the United States alone there are approximately 13 million diabetics. Of these, 2.6 million are insulin dependent diabetics. Drug & Market Dev., 4:210 (1994). Health care analysts estimate that diabetes costs \$92 billion a year resulting from medical costs and lost productivity.

- The various forms of diabetes have been organized into a series of categories developed by the National Diabetes Data Group of the National Institutes of Health. Type I diabetes in this classification scheme includes patients dependent upon insulin to prevent ketosis. This group of diabetics was previously called juvenile-onset diabetes, brittle diabetes or ketosis-prone diabetes. Type I diabetes is caused by an autoimmune reaction that causes complete destruction of beta cells.

- Type II diabetes is classified as adult-onset diabetics. The diabetic patient may or may not be insulin dependent. Type II diabetes can be caused by a number of factors. For most mammals with Type II diabetes, the beta islet cells are defective in the secretion of insulin.

- There are many therapies currently used to treat diabetes, however, each has its limitations. The major problem confronting most patients with diabetes mellitus is that currently available therapies fail to prevent the complications of the disease process. The most common method of treating Type I diabetes in mammals is providing an endogenous

1 source of insulin such as porcine, bovine or human  
insulin. Insulin injection therapy prevents severe  
hyperglycemia and ketoacidosis, but does not  
completely normalize blood glucose levels. This  
5 treatment further fails to prevent the complications  
of the disease process, including premature vascular  
deterioration. Premature vascular deterioration is  
the leading cause of morbidity among diabetic  
patients. Furthermore, complications resulting from  
10 long-term diabetes include renal failure, retinal  
deterioration, angina pectoris, arteriosclerosis,  
myocardial infarction and peripheral neuropathy.

A second method of treating diabetes is by  
transplanting the pancreas in conjunction with the  
15 administration of chronic nonspecific  
immunosuppression agents. This treatment is usually  
given to an individual who has advanced diabetes, such  
as an individual with kidney failure. Whole pancreas  
transplantation can be successfully done with a 75%  
20 one year survival rate, but surgical transplantation  
of the pancreas is very difficult. Furthermore, since  
the entire organ must be donated, the only practicable  
source is a deceased donor. In addition, when  
cyclosporine, the most common immunosuppressive drug  
25 used for organ transplants, is administered in a  
dosage necessary to suppress the immune response, the  
drug inhibits pancreatic cell function. Furthermore,  
the steroids that are often administered with an organ  
transplant often cause the patient to become diabetic.

30 A third treatment involves transplanting  
islet of Langerhans cells into the diabetic patient.

- 1 However, islet transplantation has been generally  
unsuccessful due to the aggressive immune rejection of  
islet grafts. (Gray, 1991, Immunology Letters 29:153;  
Jung et al., 1990, Seminars in Surgical Oncology  
5 6:122). In particular, successful transplantation of  
isolated pancreatic islet cells has been very  
difficult to achieve due to the chronic administration  
of immunosuppressive drugs required to prevent organ  
rejection of the cells following transplantation.
- 10 These dosages of immunosuppressive drugs can cause  
increased susceptibility to infection, hypertension,  
renal failure and tumor growth. Furthermore, unlike  
most organ transplants, islet cells must grow their  
own blood supply following implantation in the host in  
15 order for the cells to survive. Conventional  
transplantation techniques do not provide the  
necessary factors to stimulate the production of new  
blood vessels.

Thus, to successfully transplant cells in a  
20 mammal, it is necessary that the cellular transplants  
are not rejected by the recipient and have the  
capacity to grow upon transplantation. As a  
commercial reality, it is further necessary that a  
sufficient quantity of cells are available for  
25 transplantation. Traditionally, the number of  
cellular transplants have been limited by the  
inability to adequately collect and store a sufficient  
number of cells for transplantation. Conventional  
storage techniques, such as cryopreservation, often  
30 damage a large quantity of the stored cells. Porcine  
islet cells, for example, are extremely fragile and

1 easily dissociate into fragments or single cells upon  
thawing.

The present invention alleviates many of the  
problems associated with the current therapies for  
5 chronic diseases that destroy the functional cells of  
vital organs. Specifically, the present invention  
provides a method of creating systemic tolerance to  
subsequent transplants in the mammal. Furthermore,  
the present invention solves the problems associated  
10 with the conventional therapies for diabetes mellitus,  
by providing a method of transplanting pancreatic  
islets cells into a diabetic mammal, whereby the  
cellular transplants produce insulin in the diabetic  
mammal. The present inventor has previously  
15 demonstrated extended functional survival of islet  
cells allografts and xenografts in the testis.  
(Selawry et al., 1989, Diabetes 38:220.) It has been  
surprisingly discovered in accordance with the present  
invention that an immunologically privileged site can  
20 be created in a mammal by transplanting Sertoli cells  
to a nontesticular site in a mammal. The newly  
created immunologically privileged site allows the  
transplantation and survival of cells that produce  
biological factors useful in the treatment of  
25 diseases, especially diabetes. In addition to  
creating an immunologically privileged site, the  
Sertoli cells produce cell stimulatory factors which  
enhance the maturation, proliferation and functional  
capacity of cells. Sertoli cells have further been  
30 found to enhance the recovery rate and viability of

1 mammalian cells stored by techniques such as  
cryopreservation.

SUMMARY OF THE INVENTION

5 The present invention relates to a method of  
treating a disease that results from a deficiency of a  
biological factor in a mammal which comprises  
administering Sertoli cells and cells that produce the  
biological factor. In a preferred embodiment, the  
10 biological factor is a hormone.

In a more preferred embodiment, the disease  
is diabetes mellitus, the factor producing cells are  
pancreatic islet cells and the factor is insulin.

In yet another embodiment the cells that  
15 produce the biological factors are cells that have  
been genetically engineered, for example by  
transformation with a nucleic acid that expresses the  
biological factor.

The present invention further relates to a  
20 method of treating diabetes mellitus in a mammal  
comprising administering pancreatic islet cells and  
Sertoli cells. In a preferred embodiment the Sertoli  
cells and islet cells are administered by  
transplantation. The Sertoli cells may be isolated  
25 from a mammal or they may be derived from a Sertoli  
cell line, in accordance with the present invention.

Another aspect of this invention is directed  
to a method of creating an immunologically privileged  
site and producing cell stimulatory factors in a  
30 mammal.

1           A further aspect of the present invention is directed to a method of creating systemic tolerance to a subsequent transplant in a mammal by transplanting Sertoli cells prior to said subsequent transplant.

5           Still a further aspect of the present invention provides a method of enhancing the maturation, proliferation and functional capacity of cells in tissue culture by co-culturing these cells with Sertoli cells.

10           A method of enhancing the recovery rate and viability of frozen mammalian cells and in particular factor producing cells, in tissue culture by co-culturing these cells with Sertoli cells is further provided by the invention described herein.

15           Another aspect of the present invention is directed to a method of co-localizing, e.g., encapsulating the biological factor producing cells, e.g., islet cells, with Sertoli cells and to the use of the co-localized product for enhancing long-term  
20 immunoprotection and nutritional survival of islets and for the treatment of diabetes.

          Yet another embodiment of the present invention provides a pharmaceutical composition comprising Sertoli cells and cells that produce a  
25 biological factor. In a preferred embodiment the pharmaceutical composition comprises Sertoli cells and pancreatic islet cells and a pharmaceutically acceptable carrier.

          The present invention further provides a  
30 compartmentalized kit containing Sertoli cells and cells that produce a biological factor. An article of

- 1 manufacture comprising a packaging material and  
Sertoli cells contained within the packaging is also  
provided.

5 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the glucose responses to oral  
sustacal tolerance tests done on the monkey "Lucky" at  
intervals before pancreatectomy (Lucky-pre); after  
pancreatectomy but prior to transplantations (Lucky-  
10 post); and at intervals following transplantation (143  
days, 730 days and 930 days, respectively).

Figure 2 shows the C-peptide responses to an  
oral sustacal tolerance test at the same time  
intervals as depicted in Figure 1.

15 Figure 3 shows the glucose responses to oral  
sustacal tolerance tests in the monkey "Oscar".

Figure 4 shows the C-peptide responses in  
the same animal and at the same intervals depicted for  
Figure 3.

20 Figure 5a and 5b show the effect of  
intratesticular islet allografts on serum glucose  
levels and the insulin responses to oral glucose in  
spontaneously diabetic BB/Wor dp rats. Figure 5a  
shows the plasma glucose (mg/dl) concentrations in  
25 response to the oral glucose administration of 2 g/kg  
of a 50% glucose solution in three groups of rats:  
untreated control Sprague Dawley, transplanted  
diabetic BB/Wor dp, and insulin treated diabetic  
BB/Wor dp rats. Figure 5b shows the serum insulin  
30 levels in response to the same dose of oral glucose in

1 untreated control Sprague Dawley, and in transplanted  
BB/Wor dp rats.

Figures 6a and 6b show the effect of  
intratesticular islet allografts on plasma glucagon  
secretory responses to oral glucose and a combination  
5 of glucose plus glipizide in spontaneously diabetic  
BB/Wor dp rats. Figure 6a shows the plasma glucagon  
responses to the oral administration of 2 g/kg of a  
50% glucose solution in three groups of rats:  
10 untreated control Sprague Dawley, transplanted  
diabetic BB/Wor dp, and insulin treated diabetic  
BB/Wor dp rats. Figure 6b shows the plasma glucagon  
responses to the oral administration of 7 mg/kg of  
glipizide and 2 g/kg of a 50% glucose solution,  
15 administered 30 minutes later, in three groups of  
rats: untreated control Sprague Dawley, transplanted  
diabetic BB/Wor dp, and insulin treated diabetic  
BB/Wor dp rats. Data points are mean  $\pm$  SE of eight  
animals in each group.

20 Figure 7 shows a light micrograph of the  
pancreatic islets of Langerhans and the isolated rat  
Sertoli cells transplanted into the renal subcapsular  
space of a diabetic rat.

Figure 8 shows an electron micrograph of an  
25 individual cell within the transplanted islet.

Figure 9 shows an electron micrograph of the  
fine structure of the extra-islet cells labeled "S" in  
Figure 7.

Figure 10 shows the effect of  
30 transplantation of piglet islets and Sertoli cells  
underneath the renal capsule on the mean daily urine

- 1 output of seven grafted female rat recipients. Each  
bar represents the mean daily urine output over a ten-  
day period following transplantation.

- Figure 11 shows the effect of the  
5 transplantation of piglet islets and Sertoli cells  
underneath the skin on the mean daily urine volumes of  
three rats over a 50-day period.

- Figure 12 shows the light photomicrograph of  
pig islets of Langerhans and rat Sertoli cells  
10 transplanted into the renal subcapsular space of a  
diabetic rat. IL shows the presence of islands of  
beta cells (IL) surrounded by an infiltration of small  
lymphocytes underneath the renal capsule (K); B (upper  
left) shows at higher magnification that the islands  
15 (IL) consist of beta cells and B (lower right) shows  
that beta cells contain characteristic insulin  
granules.

#### DETAILED DESCRIPTION OF THE INVENTION

- 20 The present invention is directed to a  
method of treating a disease that results from a  
deficiency of a biological factor in mammals which  
comprises administering to a mammal Sertoli cells and  
a therapeutically effective amount of cells that  
25 produce the biological factor. As defined by the  
present invention, a biological factor is a protein or  
nonprotein compound that is necessary for cellular  
metabolism and homeostasis. In a preferred  
embodiment, the biological factor is a hormone.  
30 Hormone producing cells which can be administered  
using the method described in the present invention

1 include, for example, pancreatic islet of Langerhans,  
pituitary, liver, parathyroid, thyroid and ovarian  
cells.

5 In accordance with the present invention,  
the Sertoli cells and the cells that produce the  
biological factor can be from the same species as the  
mammal to be treated or from a different species.  
Further, the Sertoli cells and the cells that produce  
10 the biological factor need not be derived from the  
same species. It has been demonstrated in accordance  
with the present invention that Sertoli cells from  
pigs in conjunction with islet of Langerhans from pigs  
can be used in the treatment of diabetes mellitus in  
rats. In a preferred embodiment the Sertoli cells are  
15 bovine, porcine or human.

Sertoli cells, which are the predominant  
cells of male testes, used in the method described by  
the present invention can be separated from other  
testicular cells such as Leydig cells, peritubular  
20 cells and germ cells, using conventional techniques.  
For example, the testes of a male mammal, such as a  
boar or ram, are first collected by castration. The  
testes are then chopped into several pieces and  
subsequently washed by centrifugation.

25 Testicular Leydig cells can be removed from  
the tissue suspension using digestion agents such as  
trypsin and DNase. The remaining cell suspension is  
then washed by centrifugation several times. The  
pellet is resuspended in collagenase, incubated and  
30 washed by centrifugation to eliminate peritubular  
cells within the testes. Testicular germ cells can be

1 removed by incubating the pellet with hyaluronidase  
and DNase. After several washings by centrifugation,  
the Sertoli cells are collected to transplant using  
the method of the present invention.

5 In accordance with the present invention,  
the Sertoli cells may be obtained by various  
methodologies which establish a line of cells derived  
from primary cultures of mammalian Sertoli cells. In  
one embodiment the Sertoli cells are immortalized with  
10 a chemical or viral transformant, e.g., a temperature-  
sensitive mutant of the SV40 virus that allows  
propagation and promotes differentiation of the cells.  
In another embodiment Sertoli cells are isolated from  
mammalian tissue by conventional means using various  
15 hydrolytic enzymes such as collagenase, hyaluronidase,  
and the like. The cells are further isolated from the  
tissue by such conventional methods as filtering and  
centrifugation to obtain a purified Sertoli cell  
population. The isolated and purified Sertoli cells  
20 are next incubated and conventionally immortalized  
under conditions known in the art such as treating  
said cells with a chemical, that transforms the DNA  
thereof, e.g. a mutagen. Examples include N-  
nitrosylmethylureas, nitrous acid, hypoxanthine,  
25 nitrosamines (see, Freshney, I.R. in Culture of Animal  
Cells, A Manual of Basic Technique, 3 ed., Chapter 15,  
Wiley-Liss, New York). Alternately, the isolated  
purified sertoli cells are incubated in a virus-  
containing medium consisting of, e.g., SV40 virus or  
30 polyoma virus and a conventional growth medium such as  
F12/DMEM, for sufficient time to propagate the Sertoli

1 cells, which are then isolated from the virus. If an  
infectious virus cell is being utilized, then it is  
preferred that the virus be attenuated by techniques  
known in the art. The Sertoli cells may be isolated  
5 from the virus or chemical by conventional techniques  
employing hydrolytic enzymes. To verify that the  
Sertoli cells are produced by this methodology, the  
isolated Sertoli cells are optionally screened for the  
expression of an appropriate isolate for cloning,  
10 e.g., on the basis of expression of mRNAs encoding  
Sertoli cell-secreted proteins.

In accordance with the present invention, a  
biological factor is a protein or nonprotein compound  
that is absent, deficient or altered in a disease  
15 state. Cells that produce a biological factor can be  
isolated, for example, by first surgically removing  
the tissue that produces the factor from a mammal.  
This tissue is subsequently chopped and digested using  
conventional techniques. For example, the tissue can  
20 be digested using a collagenase digestion. The  
particular factor producing cells can subsequently be  
collected from the digestion mixture using a  
separation gradient such as a Ficoll gradient. The  
factor producing cells are then grown in tissue  
25 culture in serum using conventional techniques.

In accordance with the present invention,  
the factor producing cells may be co-cultured with  
Sertoli cells in tissue culture. Furthermore, factor-  
producing mammalian cells may be co-cultured, co-  
30 localized or co-transplanted with Sertoli cells to  
enhance the maturation, proliferation and functional

1 capacity of the mammalian cells. It has been  
demonstrated in accordance with the present invention  
that the maturation of porcine islet cells was  
enhanced when these cells were co-cultured with  
5 Sertoli cells as evidenced by both the structural  
integrity and functionality of the porcine islet cells  
compared to the islet cells cultured without Sertoli  
cells. Thus, maturation is defined by the present  
invention as the process by which a cell develops and  
10 becomes functional. The enhanced proliferation of  
porcine islet cells co-cultured with Sertoli cells is  
evidenced by the larger number of viable, insulin  
producing cells compared to porcine islet cells  
cultured without Sertoli cells. Proliferation as used  
15 herein, is defined as a process in which cells  
multiply. The enhanced functional capacity of porcine  
islet cells cultured with Sertoli cells is evidenced  
by the greater capacity of the co-cultured islet cells  
to respond to glucose and glucose plus Forskolin as  
20 insulin secretagogues. Functional capacity is defined  
as the ability of a cell to respond the biological  
environment and to generate various chemical and  
biological substances in response to the various  
substances present in the biological environment (e.g.  
25 when islet cells produce insulin in the presence of  
glucose).

Mammalian cells which can be co-cultured,  
co-localized or co-transplanted with Sertoli cells as  
described by the present invention include, for  
30 example, germ cells, such as sperm cells, oocytes,  
ovarian cells and zygotes; endocrine cells, such as

1 pancreatic islet cells, chromaffin, thyroid cells,  
hepatocytes, parathyroid cells, Leydig cells,  
follicular cells; hybridoma cells; recombinantly  
transformed cells; epithelial cells; nerve cells and  
5 epidermal cells. In a preferred embodiment, the  
mammalian cell is a germ cell or endocrine cell.  
Cells grown in tissue culture can be transplanted into  
a mammal in conjunction with the Sertoli cells using  
the methods of the present invention. In accordance  
10 with the present invention, factor producing cells may  
be stored using a variety of conventional techniques,  
such as cryopreserving the cells prior to growth in  
tissue culture for subsequent transplantation. It has  
been observed in accordance with the present  
15 invention, that Sertoli cells co-cultured, co-  
localized or co-transplanted with mammalian cells, and  
in particular factor producing cells such as islet  
cells, enhance the recovery rate and viability of the  
mammalian cells in tissue culture and in particular,  
20 enhance the recovery rate and viability of cells that  
have been previously stored using techniques such as  
cryopreservation. Moreover, when co-localized with  
factor producing cells, such as islet cells, Sertoli  
cells provide immunoprotection and nutritional support  
25 when the two cell types are proximally located.  
Sertoli cells protect the factor producing cells, such  
as islets, from, inter alia, macrophages, proteins,  
lymphokines (e.g., IL-1) and toxic factors released by  
activated lymphocytes. Sertoli cells provide  
30 nutritional support for islets through Sertoli  
secreted growth factors, e.g., IGF (insulin-like

1 growth factor), EGF (epidermal growth factor) and  
transferrin, thereby permitting the factor-producing  
cells to survive longer than when the Sertoli cells  
are not present.

5 In a preferred embodiment the factor is a  
hormone, and the hormone producing cells are isolated  
from a tissue source as described above. For example,  
insulin-producing cells are isolated from the  
pancreas. In another preferred embodiment, the factor  
10 producing cells are provided by transforming suitable  
host cells with a nucleic acid capable of expressing  
the factor of interest. Transformed cells are  
provided by methods known to one of ordinary skill in  
the art, and can be found in a myriad of textbooks and  
15 laboratory mammals, including Sambrook et al. (1989)  
Molecular Cloning: A Laboratory Mammal, Cold Spring  
Harbor Laboratories, Cold Spring, New York. If  
necessary, the nucleic acid encoding the factor of  
interest can be adapted by methods known to one of  
20 ordinary skill in the art to effect secretion of the  
factor from the transformed cell. The utilization of  
Sertoli cells in conjunction with the factor producing  
cells in accordance with the method of the present  
invention allows the production of an immunologically  
25 privileged site and production of cell stimulatory  
factors in the treated mammal.

The administration of factor producing cells  
and Sertoli cells into a mammal is accomplished by  
conventional techniques. In a preferred embodiment,  
30 administration is by transplantation and the factor  
producing cells are injected into the mammal

1 concurrently with or immediately after the injection  
of the Sertoli cells into the same site. In another  
embodiment, Sertoli cells and cells producing the  
biological factor are co-localized and administered to  
5 a mammal. As an example, islets and Sertoli cells are  
co-encapsulated and injected into the mammal  
intraperitoneally. In another embodiment, co-  
localized islets and Sertoli cells are transplanted,  
injected or provided into a biological or non-  
10 biological biocompatible material (biomaterial).  
Examples of a biological biocompatible material  
include an isolated segment of small intestine with  
intact circulation, a pouch (e.g. an omental pouch or  
a gastric pouch etc.), a biocompatible polymeric  
15 scaffold, a polymeric sponge or matrix, and the like,  
prepared pursuant to conventional techniques. On the  
other hand, a non-biological, biocompatible material  
includes reticulated thermoplastics such as  
acrylonitrile vinyl chloride copolymer (PAN-PVC), and  
20 the like. The biomaterial may be conventionally  
implanted in a mammal. In accordance with the present  
invention, an exogenous biological factor may be  
administered following the transplantation of factor  
producing cells and Sertoli cells until the  
25 transplanted cells produce a therapeutically effective  
amount of the biological factor. For the treatment of  
diabetes, for example, insulin may be administered  
following the transplantation of pancreatic islet  
cells and Sertoli cells until the transplanted islet  
30 cells produce a therapeutically effective amount of  
insulin.

1           The Sertoli cells and factor producing cells  
of the present invention can be transplanted or co-  
localized using any technique capable of introducing  
the cells into the mammal such as parenteral  
5 administration, subcutaneous administration following  
surgical exposure to a desired site, biocompatible  
scaffold, sponge or matrix delivery or intraperitoneal  
administration. Prior to transplantation, the  
recipient mammal is anesthetized using local or  
10 general anesthesia according to conventional  
technique. In a preferred embodiment the mammal to be  
treated is human. In another embodiment the present  
method of treating disease further comprises  
administering an immunosuppressive agent such as, for  
15 example, cyclosporine, tacrolimus, despergualin and  
monoclonal antibodies to, e.g., T cells. In a  
preferred embodiment the immunosuppressive agent is  
cyclosporine. In another preferred embodiment  
cyclosporine is administered at a dosage of from 0.5  
20 mg to 200 mg/kg body weight. In a most preferred  
embodiment cyclosporine is administered at a dosage of  
from 5 mg to 40 mg/kg body weight.

It has been discovered in accordance with  
the present invention that administration of Sertoli  
25 cells results in the creation of an immunologically  
privileged site in the treated mammal and in the  
production of cell stimulatory factors. An  
immunologically privileged site as defined by the  
present invention is a site in the mammal where the  
30 immune response produced in response to the  
transplanted cells is suppressed due to immuno-

1 suppressive agents produced by Sertoli cells.  
Immunologically privileged sites are characterized by  
an available blood supply to provide nourishment for  
the transplanted cells and a dense tissue to keep the  
5 transplanted cells within close proximity of each  
other. Examples of immunologically privileged sites  
as defined by the present invention include the renal  
subcapsular space, subcutaneous facie, the brain and  
the hepatic portal vein. Cell stimulatory factors are  
10 defined by the present invention as factors that  
enhance the viability of mammalian cells. For  
example, it has been shown in accordance with the  
present invention that Sertoli cells increase the rate  
at which the transplanted factor producing cells  
15 vascularize in the transplanted site (i.e. promote  
angiogenesis). Further, it has been shown by the  
present invention that these cell stimulatory factors  
enhance the maturation, proliferation and functional  
capacity of cells transplanted with Sertoli cells. It  
20 is therefore indicated that the Sertoli cells produce  
cell stimulatory factors which enhance of viability of  
mammalian cells as evidence, for example, by the  
increased vascularization rate of the transplanted  
islet cells. As used herein, viability denotes the  
25 number of living cells in a preparation.

In a preferred embodiment, the present  
invention describes a method of treating diabetes  
mellitus by transplanting islet of Langerhans in  
conjunction with Sertoli cells to create an  
30 immunologically privileged site. Allografts as used  
in the present invention describes the transfer of

1 tissues or cells between two genetically dissimilar  
mammals of the same species. The term xenografts in  
the present invention describes the transfer of  
tissues or cells between two mammals of different  
5 species.

The transplanted islet of Langerhans cells  
and Sertoli cells used in the method described by the  
present invention can be prepared using any number of  
conventional techniques. For example, islet of  
10 Langerhans cells can be prepared from the pancreas of  
several mammals of the same species. The pancreases  
are pooled together, chopped up and digested using  
collagenase. The islet of Langerhans cells can be  
further isolated using conventional gradients. Once  
15 isolated, the islet cells can be grown in culture and  
then transplanted in conjunction with Sertoli cells to  
create an immunoprivileged site.

Sertoli cells used in the method described  
by the present invention can be derived from primary  
20 cultures of mammalian Sertoli cells according to the  
methods known to one skilled in the art including the  
method of e.g. Roberts et al. (1995) Biology of  
Reprod. 53:1446-1453, the contents of which is  
incorporated herein by reference, or the Sertoli cells  
25 can be isolated from mammalian male testes. To  
collect the islet cells, the testes are first chopped  
into several pieces and then washed by centrifugation.  
Leydig cells, present in the crude mixture, can be  
removed from the tissue suspension using digestion  
30 agents such as trypsin and DNase. The remaining cell  
suspension is then washed by centrifugation several

1 times. Following, the pellet may be resuspended in  
collagenase, incubated and washed by centrifugation to  
eliminate peritubular cells within the testes.  
Testicular germ cells can be removed by incubating the  
5 pellet with hyaluronidase and DNase. After several  
washings by centrifugation, the Sertoli cells for  
transplantation can be collected.

The Sertoli cells can be transplanted to  
create an immunoprivileged site within a mammal using  
10 a variety of techniques. For example, after the  
mammal is anesthetized, the Sertoli cells can be  
injected into a tissue mass, thereby creating an  
immunoprivileged site. The Sertoli cells and factor  
producing cells of the present invention can be  
15 combined using the techniques capable of co-localizing  
the cells such as microencapsulation inside  
biocompatible membranes, hydrogels, or reticulated  
thermoplastics, for example. The co-localized cells  
can subsequently be injected, transplanted or provided  
20 into a tissue mass subcutaneously or into a pouch,  
e.g. an intestinal pouch, an omental pouch, a gastric  
pouch, or a biocompatible polymeric scaffold, sponge  
or matrix consisting of, e.g., polylactic acid. Once  
injected, transplanted, or provided, the co-localized  
25 product is used to treat diseases caused by a  
deficiency of the biological factor. For example, the  
Sertoli cells and islet cells in combination are  
useful in treating diabetes mellitus.

Sertoli cells are administered in an amount  
30 effective to provide an immunologically privileged  
site. Such an effective amount is defined as that

1 which prevents immune rejection of the subsequently or  
co-administered cells that produce the biological  
factor. Immune rejection can be determined for  
example histologically, or by functional assessment of  
5 the factor produced by the cells.

The present invention further provides a  
method of creating systemic tolerance to a subsequent  
transplant in a mammal by transplanting Sertoli cells  
prior to said subsequent transplant as described  
10 herein. A transplant as used herein is a mammalian  
cell, tissue, organelle or organ that is removed from  
one mammal and placed in the same or different mammal.  
The subsequent transplant may be made in the same site  
or a secondary site. A secondary site as used herein,  
15 is a transplantation site in the mammal different from  
the initial transplantation site. Systemic tolerance  
is demonstrated by various biological phenomena. For  
example, systemic tolerance results in a diminished  
destructive immune response to a subsequent allograft  
20 or xenograft in a mammal without the administration of  
prolonged immunosuppressive agents or the co-  
transplantation of Sertoli cells. In accordance with  
the present invention, the allograft or xenograft may  
be any type of transplant, including cells, tissues,  
25 organelles or an organ. The types of cells which may  
be transplanted in accordance with the methods  
described by the present invention include, for  
example, endocrine cells, bone marrow cells,  
hepatocytes or liver cells, nerve cells or brain  
30 cells, and islet cells (fetal, neonatal or adult).  
The types of tissues, organelles or organs which may

1 be transplanted in accordance with the methods  
described by the present invention include, for  
example, heart, kidney, pancreas, liver, skin,  
ligaments, tendons and cartilage.

5 As demonstrated by the present invention, a  
mammal may be tolerized (i.e. systemic tolerance may  
be achieved) by a variety of procedures. For example,  
systemic tolerance may be achieved by transplanting an  
allograft or xenograft with Sertoli cells and  
10 subsequently transplanting the same type of allograft  
or xenograft without Sertoli cells or a prolonged  
administration of immunosuppressive agents. Systemic  
tolerance may also be achieved by transplanting an  
allograft of any cell, tissue, organelle or organ  
15 without Sertoli cells or prolonged immunosuppressive  
agents following an initial transplantation of an  
allograft with Sertoli cells. In a preferred  
embodiment Sertoli cells are administered in amounts  
ranging from  $10^1$  to  $10^{10}$  cells. In a more preferred  
20 embodiment,  $10^5$  to  $10^{10}$  cells are administered.

The cells producing the biological factor  
are administered in a therapeutically effective  
amount. The ordinary skilled artisan can determine  
the appropriate amount of cells producing the  
25 biological factor by methods known in the art. The  
amount of cells is dependent upon the amount of factor  
being produced by the cells and the known  
therapeutically effective amount of the factor  
necessary to treat the disease. For example, 1 to  
30 1000 islet cells per gram body weight can be  
administered to treat diabetes using allografts, 20 to

1 1000 islets per gram body weight are administered  
using xenografts. In another preferred embodiment, 5  
to 100 islet cells per gram body weight are  
administered to treat diabetes. In a most preferred  
5 embodiment, 5 to 20 islet cells per gram body weight  
are administered, using allografts and 100-1000 islet  
cells per gram body weight are administered for  
xenografts.

In another embodiment the present method of  
10 treating diabetes further comprises administering an  
immunosuppressive agent such as, for example,  
cyclosporine, tacrolimus, desfergualin and monoclonal  
antibodies to, e.g., T cells. In a preferred  
embodiment the immunosuppressive agent is  
15 cyclosporine. In another preferred embodiment  
cyclosporine is administered at a dosage of from 0.5  
mg to 200 mg/kg body weight. In a most preferred  
embodiment cyclosporine is administered at a dosage of  
from 5 mg to 40 mg/kg body weight.

20 More generally, the immunosuppressive agent  
can be administered for a time sufficient to permit  
the transplanted islets to be functional. This period  
extends from the point prior to or immediately  
following the transplantation of the islets to the  
25 point at which the cells are capable of producing  
therapeutically effective amounts of insulin. In a  
preferred embodiment, the sufficient period of time to  
administer an immunosuppressive agent is about 40 to  
about 100 days following transplantation of the  
30 islets. In a more preferred embodiment, the  
sufficient period of time is about 50-60 days.

1           A preferred embodiment of this invention is  
directed to a method of treating Type I and Type II  
diabetes mellitus by transplanting islet of Langerhans  
in conjunction with Sertoli cells into the renal  
5   subcapsular space.

Unlike the therapies for diabetes described  
in the prior art, the method of treating diabetes  
described by the present invention prevents the  
complications of the disease process and does not  
10   result in the adverse side effects associated with  
conventional diabetes therapy. Furthermore, the  
method of transplanting islet cells described by the  
present invention provides the necessary factors for  
angiogenesis, growth enhancing and increased  
15   functional capacity of the islet transplants.

A method of creating an immunologically  
privileged site in a mammal is further described by  
the present invention. An immunologically privileged  
site is created by transplanting isolated Sertoli  
20   cells into a mammal in an amount effective to create  
an immunologically privileged site. In a preferred  
embodiment,  $10^1$  to  $10^{10}$  cells are administered. In a  
more preferred embodiment,  $10^5$  to  $10^{10}$  cells are  
administered. In a preferred embodiment the Sertoli  
25   cells are transplanted into the renal subcapsular  
space or subcutaneous facie by injection. In a  
preferred embodiment the mammal is a human and the  
Sertoli cells are human or porcine.

A further aspect of the present invention is  
30   directed to a method of enhancing the recovery rate  
and viability of frozen mammalian cells in tissue

1 culture comprising co-culturing the frozen mammalian  
cell with Sertoli cells. As shown in accordance with  
the present invention, Sertoli cells produce cell  
stimulatory factors which enhance the recovery rate  
5 and viability of mammalian cells previously frozen.  
Mammalian cells may be frozen using a widely  
conventional techniques, including, for example,  
cryopreservation.

Further contemplated in accordance with the  
10 present invention is a method of enhancing the  
recovery and proliferation of ex vivo cells comprising  
co-culturing said cells with Sertoli cells for a time  
and under conditions sufficient to achieve said  
enhanced recovery and proliferation.

15 Another aspect of the present invention  
provides a pharmaceutical composition comprising  
Sertoli cells and cells producing a biological factor  
and a pharmaceutically acceptable carrier. In a  
preferred embodiment the composition comprises Sertoli  
20 cells and islet of Langerhans cells and a  
pharmaceutically acceptable carrier. A further  
preferred embodiment of the present invention  
comprises using porcine, bovine or human Sertoli cells  
and porcine, bovine or human islet of Langerhans  
25 cells. As used herein, a pharmaceutically acceptable  
carrier includes any and all biological and non-  
biological biocompatible membrane materials. A  
pharmaceutically acceptable carrier also includes any  
conventional solvents, dispersion media, coatings,  
30 antibacterial and antifungal agents, isotonic agents

1 and the like. The use of such media and agents is well-known in the art.

The present invention further contemplates a pharmaceutical composition comprising Sertoli cells  
5 and a pharmaceutically acceptable carrier. This pharmaceutical composition, upon administration to a mammal, can be used to treat a variety of diseases, such as for example, autoimmune diseases. Accordingly, the present invention is further directed  
10 to treating an autoimmune disease in a mammal comprising administering a therapeutically effective amount of Sertoli cells to the mammal.

The present invention is further directed to a method of enhancing the recovery and proliferation  
15 of ex vivo cells comprising culturing said cells with a culture media from a tissue culture containing Sertoli cells for a time and under conditions sufficient to achieve said enhanced recovery and proliferation. As contemplated by the present  
20 invention, Sertoli cells are cultured using a conventional tissue culture media as described herein for a time and under conditions sufficient for the Sertoli cells to produce, for example, cell stimulatory factors. The Sertoli cells are then  
25 removed from the culture media and the culture media is used in subsequent tissue cultures, for example, as a culture media for sperm cells previously stored by cryopreservation.

Another aspect of the present invention is  
30 directed to methods of co-localizing biological factor producing cells, e.g., islets of Langerhans, with

1 Sertoli cells to enhance long-term immunoprotection  
and nutritional survival of transplanted factor  
producing cells, e.g., islets. The methods of co-  
localization include co-localization in an intestinal  
5 segment, pouch, e.g. an omental pouch, a gastric pouch  
or biocompatible polymeric scaffold, sponge or matrix,  
for example. The method of co-localization in an  
intestinal segment comprises:

- (a) isolating a segment of mammalian small  
10 intestine with intact mucosa and intact circulation;
- (b) removing the mucosal layer of the small  
intestine and closing the ends of the isolated  
segment;
- (c) implanting biological factor producing  
15 cells and Sertoli cells into the isolated segment; and
- (d) fixing the isolated segment to the  
small intestine.

Methods of co-localizing biological factor  
producing cells, e.g., islets with Sertoli cells in  
20 pouches, such as omental or gastric pouches are also  
contemplated by the present invention as described by  
Amiri, et al. (1990) Arch. Surg. 125:1472-1474 and  
Bayat, et al. (1995) Surg. Res. Commun. 17:87-91, both  
of which are incorporated herein by reference.

25 Procedures for co-localizing islets and Sertoli cells  
in biologically compatible pouches are general and  
conventionally employed on a case-by-case basis by the  
skilled artisan in accordance with the present  
invention.

30 Procedures for co-localizing islets and  
Sertoli cells in polymeric scaffolds are readily

1 appreciated by the skilled artisan. It is preferred  
that the polymeric templates used in accordance with  
the present invention are biodegradable and comprise a  
polyvinyl alcohol, e.g., poly-L-lactic acid.

5 Polyvinyl alcohol based templates provide sufficient  
porosity to permit rapid tissue ingrowth and  
prevascularization before cell transplantation. In  
essence, the polymeric template employed in connection  
with the present invention acts as a matrix or sponge  
10 permitting the ingrowth of blood vessels and tissue  
which facilitates the co-localization of, e.g., islets  
and Sertoli cells by providing ready access to a  
nutrient-rich blood supply.

It is preferred that the Sertoli cells are  
15 provided in an amount ranging from about  $10^1$  to about  
 $10^{10}$  cells. In a more preferred embodiment, Sertoli  
cells are provided in an amount ranging from about  $10^4$   
to about  $10^{10}$  cells. In yet another preferred  
embodiment, the factor producing cells are pancreatic  
20 islets. The islets are provided in a preferred amount  
of about 5 to about 200 cells per gram of body weight,  
and in a more preferred amount of about 5 to about 100  
cells per gram of body weight.

A further aspect of the present invention is  
25 directed to a method of encapsulating biological  
factor producing cells, e.g., islets, with Sertoli  
cells to enhance long-term immunoprotection and  
nutritional survival of transplanted islets. The  
method of encapsulation comprises:

30 (a) suspending a pharmaceutically effective  
amount of biological factor producing cells, e.g.,

- 1 islets, and Sertoli cells in combination with a  
gelling effective amount of a first water soluble  
gelling agent in an aqueous medium which is  
physiologically compatible with the cells and  
5 extruding the islet/Sertoli cell/gelling agent mixture  
to form a droplet containing the islets and Sertoli  
cells;

- (b) subjecting the product of step (a) to  
an effective amount of network forming cations to form  
10 discrete capsules of sufficient size to encapsulate  
the islets and Sertoli cells together;

(c) forming a semipermeable membrane around  
the capsules to obtain a single-walled bead  
encapsulating the cells; and

- 15 (d) contacting the single-walled bead with  
a gelling effective amount of a second gelling agent  
so as to form a second semi-permeable membrane  
encapsulating the product of step (c).

- The gelling agent may be any water-soluble  
20 material which can be gelled to form a bead. A  
preferred gelling agent is a water soluble, natural or  
synthetic polysaccharide gum such as an alkali metal  
alginate. A preferred gum is sodium alginate. Other  
gums which may be used include guar gum, gum arabic,  
25 carrageenan, pectin, tragacanth gum, xanthan gum or  
their acidic fractions.

- In a preferred embodiment, the Sertoli cells  
and factor producing cells are encapsulated within an  
alginate polylysine-alginate semi-permeable hydrogel.  
30 It is preferred that the Sertoli cells are derived  
from bovine, porcine, and human sources and are

1 produced by a cell line in accordance with the present  
invention. It is preferred that the Sertoli cells are  
provided in an amount of from  $10^1$  to  $10^{10}$  cells. In a  
more preferred embodiment, Sertoli cells are provided  
5 in an amount of from about  $10^4$  to  $10^{10}$  cells. In yet  
another preferred embodiment the factor producing  
cells are pancreatic islets. The islets are provided  
in a preferred amount of about 5 to about 200 cells  
per gram of body weight, and in a more preferred  
10 amount of about 5 to about 100 cells per gram of body  
weight.

The procedure for the encapsulating of  
Sertoli cells with cells that produce a biological  
factor is general, and the procedure will be explained  
15 in more detail with respect to Sertoli cells and islet  
cells, which are exemplary.

In an embodiment of step (a) of the subject  
method, the first gelling agent is sodium alginate.  
It is conventionally suspended in an aqueous solution  
20 such as a buffer or saline solution containing the  
islets and Sertoli cells.

By "gelling effective amount" is meant an  
amount of a water soluble gelling agent capable of  
binding calcium ions or other ions that interact with  
25 the gelling agent to form a network. More  
specifically, the islets and Sertoli cells in  
combination are preferably suspended relative to the  
gelling agent in a ratio of about 1:20 to 20:1 (v/v)  
and more preferably 1:10 to 10:1 (v/v) and most  
30 preferably about 1:10 (v/v). Preferably, the Sertoli

- 1 cells and islets cells are present in a ratio of about  
1:1 (v/v).

The suspension is extruded by techniques commonly used in the art. It is preferred that the  
5 suspension is extruded through an air-jet needle. In a preferred embodiment, droplets containing islets and Sertoli cells in association with the alginate are produced by extrusion (1.7 ml/min) through a 22 gauge air-jet needle (air flow 5 l/min).

- 10 In an embodiment of step (b) of the subject method, the droplets are subjected to a solution of multivalent cations, such as a solution of calcium salt, e.g., calcium halide, e.g., calcium chloride solution, which form a network within said droplet.  
15 The preferred concentration is at least about 0.5% (v/v), and more preferably at least about 1% (v/v), and most preferably ranging from about 1% (v/v) to a saturated solution. In an embodiment, the droplets fall into a beaker containing a saline solution at pH7  
20 of calcium chloride solution, e.g., 10 ml 1.1% CaCl<sub>2</sub> in 0.9% saline at pH7. This process continues for a sufficient time until the negatively charged alginate droplets bind calcium and form calcium alginate gel.

- In an embodiment of step (c) of the subject  
25 method, a membrane is formed around the product of step (b) by subjecting the encapsulated product to polymers, which polymers contain substituents reactive with the gelling agents, especially the acid groups of the gelling agent. The preferred polymers are  
30 polyamine acids such as poly-L-lysine (PLL) or polyethylenimine. In a preferred embodiment, the

1 polymer is poly-L-lysine with a molecular weight of  
about 20kd. It is preferred that the polymers coat  
the product of (b) by following the procedure of  
Goosen, et al. in Biotech. Bioeng., 27: 146-150  
5 (1985), the contents of which is incorporated herein  
by reference. Without wishing to be bound, it is  
believed that positively charged poly-L-lysine  
displaces calcium ions and binds negatively charged  
alginate, producing a polyelectrolyte membrane.

10 In an embodiment of step (d) of the subject  
method the second gelling agent may be sodium alginate  
which may improve the biocompatibility of the capsule  
in a mammal. The second gelling agent may be added  
according to the methods employed by Weber, et al.  
15 U.S. Patent No. 5,227,298, incorporated herein by  
reference. The double walled semi-permeable capsules  
formulated in connection with the present invention  
have molecular weight cut-offs in the range of 50,000  
daltons and provide sustained release of factors  
20 produced by the encapsulated islets and Sertoli cells.  
The capsules comprise semi-permeable membranes which  
function to protect islets from immune responses while  
simultaneously permitting passage of biological  
factors produced by islets into the mammal. In still  
25 another embodiment of the present invention, co-  
localized, e.g., co-encapsulated islets and Sertoli  
cells are connected to a blood supply by techniques  
known in the art, thereby permitting the free flow of  
nutrients and inhibiting the influx of molecules  
30 produced by the immune system.

1           The co-localized, e.g., encapsulated cells  
producing biological factor and Sertoli cells are  
effective in treating a disease resulting from a  
deficiency of said biological factor. For example,  
5   the co-localized, e.g., encapsulated islet cells with  
Sertoli cells, are effective in treating diabetes  
mellitus. Thus a preferred embodiment of this  
invention is directed to a method of treating diabetes  
mellitus by co-localizing, e.g., co-encapsulating and  
10   transplanting islet of Langerhans into the peritoneal  
space. This method not only prevents the  
complications of the disease process, but also reduces  
the adverse effects associated with other therapies.  
This method also provides a biological factor in  
15   appropriate amounts which are released in a  
physiological manner.

          The present invention is also directed to a  
kit for treatment of a disease. In one embodiment,  
the kit is compartmentalized to receive a first  
20   container adapted to contain Sertoli cells in an  
amount effective to create an immunologically  
privileged site in a mammal, and a second container  
adapted to contain a therapeutically effective amount  
of cells that produce a biological factor that is  
25   absent or defective in the disease to be treated. In  
a preferred embodiment, the Sertoli cells are bovine,  
porcine or human and are provided in an amount of from  
 $10^1$  to  $10^{10}$  cells. In a more preferred embodiment,  
Sertoli cells are provided in an amount of from  $10^5$  to  
30    $10^{10}$  cells. In another preferred embodiment the cells  
that produce a biological factor are cells that have

1 been transformed with DNA encoding the factor. In yet  
another preferred embodiment the cells that produce  
the factor are pancreatic islet cells. The islet  
cells are provided in a preferred amount of 5 to 200  
5 cells per gram of body weight, and in a more preferred  
amount of 5 to 100 cells per gram of body weight.

The present invention further provides an  
article of manufacture comprising a packaging material  
and Sertoli cells contained within said packaging  
10 material, wherein said Sertoli cells are effective for  
creating an immunologically privileged site in a  
mammal, and wherein said packaging material contains a  
label that indicates that said Sertoli cells can be  
used for creating an immunologically privileged site  
15 in a mammal. The packaging material used to contain  
the Sertoli cells can comprise glass, plastic, metal  
or any other suitably inert material.

Unless specified to the contrary, it is to  
be understood that percentages are by volume.

20 In order to further illustrate the present  
invention, the experiments described in the following  
examples were carried out. It should be understood  
that the invention is not limited to the specific  
examples or the details described therein. The  
25 results obtained from the experiments described in the  
examples are shown in the accompanying figures and  
tables.

30

35

1

EXAMPLE 1

5 Six male Rhesus monkeys were transplanted with islet allografts in their testes to examine the survival of these transplants. The recipients were made diabetic by means of a near total pancreatectomy, followed two weeks later by an intravenous injection of 35 mg streptozotocin/kg body weight. This procedure resulted in the induction of severe diabetes melitis. Plasma glucose levels were in excess of 400 mg/dl and the animals were ketotic. Malabsorption was prevented by the oral administration of VACUOUS®, one tablet given twice daily before each meal.

10 Islets were isolated from female Rhesus monkeys. First, the pancreases of five animals were removed, pooled and chopped finely into smaller fragments. After collagenase digestion in a water bath at 37°C, the islets were separated from exocrine tissues and other cellular debris on at least two Ficoll gradients, prepared in tandem. The islets were washed three times by centrifugation in ice-cold Hanks's buffer and then handpicked and transferred in groups of 150 to biologic grade Petri dishes. Each dish contained 6 mL of culture medium CMRL-1066 supplemented with 5% fetal calf serum, glucose at a concentration of 250 mg/dL, penicillin (100 U/mL), and streptomycin (100 µ/mL). Incubation of islets were carried out at 35°C in 5% CO and air for 4 to 6 days. The islets were transferred to fresh medium at 48 hour intervals.

35

1            Viability and counting of the islets were  
facilitated by means of the uptake of the dye  
dithizone. Each monkey received an average of about  
10<sup>4</sup> islets/kg body weight injected into both testes.  
5    In the first three animals the testes were elevated  
into the abdominal cavity, whereas in the last three  
recipients the grafted organs were anchored into the  
inguinal canal. Cyclosporine (CsA) was administered,  
in varying doses to the first three grafted animals  
10 over a 30 day period, whereas the last three hosts  
were given 7 injections of CsA (20 mg/kg) on days -4  
to +3. Oral sustacal tolerance tests were done on day  
30, and then at intervals in the normoglycemic  
animals, as follows.

15           The monkeys were housed individually in  
cages and given standard monkey chow and fruit twice  
daily. In addition, a pancreatic enzyme was mixed  
with the food since the monkeys had been  
pancreatectomized to make them diabetic before  
20 transplantation.

            The night before the test, the animals were  
fasted for 12 hours. At 8 a.m. the next morning they  
were then anesthetized and prepared for the test meal.  
Sustacal was used as the test agent. Sustacal  
25 consists of a physiologic mixture of carbohydrates,  
proteins and fat which closely mimics a standard meal  
and which is a powerful stimulus for the release of  
insulin.

            Sustacal was injected directly into the  
30 stomach of the sleeping animal through a nasogastric  
tube. Blood samples were then obtained at times 0,

1 15, 30, 60, 90, 120 and 180 minutes. The samples were  
centrifuged and the serum stored at -20°C until  
measurements for insulin or C-peptide could be carried  
out. C-peptide is a very sensitive marker for beta  
5 cell function. The results are shown in Figures 1-4.

Figure 1 shows the glucose responses to oral  
sustacal tolerance tests done on the monkey "Lucky" at  
intervals before pancreatectomy (Lucky-pre); after  
pancreatectomy but prior to transplantation (Lucky-  
10 post); and at intervals following transplantation (143  
days, 730 days and 930 days, respectively).

It can be readily appreciated that the  
animal became severely diabetic after the removal of  
his pancreas (Lucky-post). Following transplantation  
15 the glucose responses were restored to normal levels  
at all of the time intervals measured (143, 730 and  
930 days following transplantation). Lucky showed no  
evidence of graft failure. With graft failure glucose  
levels would become elevated would approach those  
20 which were found following his pancreatectomy.

Figure 2 shows the C-peptide responses to an  
oral sustacal tolerance test at the same time  
intervals as depicted in Figure 1. Following his  
pancreatectomy the C-peptide responses became blunted  
25 indicating a severe diabetes. But following  
transplantation the levels were not only restored to  
normal but appeared to show a hyperresponsive pattern  
of C-peptide release and levels done on day 730 exceed  
the normal levels at all points measured. The  
30 elevated levels might be due to the fact that insulin  
released from the testis enters the systemic

1   circulation. By contrast, insulin released from the  
pancreas enters the portal vein and travels  
immediately to the liver where about 60% is broken  
down during the first passage. Insulin released into  
5   the systemic circulation reaches the liver much later,  
thus the elevated levels. As was evident with an  
investigation of the glucose concentrations, the C--  
peptide responses showed no evidence of failure 30  
months following transplantation.

10           Figure 3 shows the glucose responses to oral  
sustacal tolerance tests in the monkey "Oscar".  
Following the removal of his pancreas he became  
severely diabetic with elevated glucose levels.  
Following transplantation of islets the glucose  
15   responses became similar to those determined before  
his pancreas was removed. The glucose levels remain  
within normal levels 32 months following  
transplantation.

          Figure 4 shows the C-peptide responses in  
20   the same animal and at the same intervals depicted for  
Figure 3. The animal became very diabetic following  
the removal of his pancreas and shows blunted  
C-peptide responses as a result. Following  
transplantation and for the next 730 days the C-  
25   peptide responses were greater compared with the  
normals. on day 930 following transplantation the C-  
peptide responses have become somewhat less compared  
with the normals. Despite somewhat lower C-peptide  
levels the animal remains normoglycemic.

30           This example demonstrates that primates can  
be successfully transplanted with intratesticular

1 islet allografts without the need for sustained  
immunosuppression, and that functional integrity of  
intratesticular islet allografts is maintained for  
periods exceeding two years with no evidence of graft  
5 failure.

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**EXAMPLE 2**

This study examined insulin and glucagon secretory patterns in spontaneously diabetic bb/Wor dp rats transplanted with abdominal, intratesticular, islet grafts. Diabetic, BB/Wor dp, rats received intratesticular islet grafts from MHC-compatible BB/Wor dr rats and no immunosuppression. After a period of  $74 \pm 15$  days, of normoglycemia, three different groups (controls; BB/Wor dp, transplanted; and BB/Wor dp, insulin treated) were given the following challenges; (1) an oral glucose tolerance test (OGTT), (2) a single oral dose of glipizide, followed by an OGTT, and (3) arginine, by intravenous infusion. The results of this study are shown in Tables 1 and 2 and Figures 5 and 6.

**TABLE 1**

**Metabolic Parameters and Immunoreactive Serum Insulin and Glucagon Levels in Control and in Transplanted and Insulin Treated BB/Wor dp Rats**

20

	BB/Wor dp		
	CONTROLS	GRAFTED*	INSULIN TREATED
Plasma Glucose (mg/dl): Prior to Therapy	112 $\pm$ 5	502 $\pm$ 8+	510 $\pm$ 13+
After 2.5 Months	97 $\pm$ 4	110 $\pm$ 3	350 $\pm$ 40#
Duration p.t. OGTT (days)	75 $\pm$ 6	70 $\pm$ 11	78 $\pm$ 19
Weight Gain (g)	120 $\pm$ 6	105 $\pm$ 17	48 $\pm$ 14\$
Fasting Plasma Insulin (uU/ml)	21.9 $\pm$ 3	20.4 $\pm$ 2	ND
Fasting Plasma Glucagon (pg/ml)	37.8 $\pm$ 5.7	43.4 $\pm$ 4.6	47.4 $\pm$ 4.9

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\* Duration of normoglycemia after grafting (days) =  $279 \pm 25$

+ P < 0.0001 vs. control

# P < 0.0001 vs. grafted

\$ P < 0.02 vs. grafted

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TABLE 2

**Pancreatic and Testicular Insulin  
and Glucagon Content in Control and in  
Transplanted and Insulin Treated BB/Wor dp Rats**

5

	BB/Wor dp		
	CONTROLS	GRAFTED	INSULIN TREATED
Pancreas (mg)	1573±171	757±122	920±32
Insulin (ug/g)	66±5.03	0.58±0.18	0.76±0.12
Glucagon (ng/mg)	4.1±0.35*	49.±0.33**	6.9±0.08
Testes Fractions: (mg)	493±49.6	582±59.2	430±28.0
Insulin (ug/g)	0.0	59.70±0.49	0.0
Glucagon (ng/mg)	0.0	1.4±0.37	0.0

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\* P &lt; 0.03

\*\* P &lt; 0.08 vs. diabetic, respectively

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Figure 5 shows the effect of intratesticular islet allografts on serum glucose and insulin responses to oral glucose in spontaneously diabetic BB/Wor dp rats. Figure 6 shows the effect of intratesticular islet allografts on plasma glucagon secretory responses to oral glucose and a combination of glucose plus glipizide in spontaneously diabetic BB/Wor dp rats. This experiment demonstrates that grafted testes in spontaneously diabetic BB/Wor dp rats contain both alpha and beta cells, and that the alpha and beta cells have the capacity to respond to specific secretagogues independently.

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1 conical tube containing 50 mL of Ham's F12/DMEM media.  
The pieces were washed once by centrifugation at 800 x  
g for 2 min. The supernatant was aspirated, and the  
tissue resuspended in 40 mL of media containing 40 mg  
5 trypsin and 0.8 mg DNase in a sterile 250 mL  
Erlenmeyer flask. The flask was placed in 37°C  
oscillating incubator at 60-90 osc/min for 30 min.  
This step removed Leydig cells. The tubules were then  
transferred to a 50 mL conical tube, and centrifuged  
10 at 800 x g for 2 min. The supernatant fraction was  
aspirated, and the pellet resuspended in 40 mL of 1 M  
glycine, 2 mM EDTA containing 0.01% soy bean trypsin  
inhibitor and 0.8 mg DNase, and incubated at room  
temperature for 10 min. This step lysed any residual  
15 Leydig cells. The cells were washed by centrifugation  
for 2 min, and the step repeated twice, or until the  
media was no longer cloudy. The pellet was  
resuspended by gentle homogenization with a glass  
Pasteur pipet in 40 mL of media containing 20 mg  
20 collagenase in an Erlenmeyer flask, and incubated at  
37°C for 5 min with 60-90 osc/min. The cell  
suspension was centrifuged at 800 x g for two min, and  
the pellet resuspended by gentle homogenization with a  
Pasteur pipet in 40 mL media containing 40 mg  
25 collagenase and 0.2 mg DNase, and incubated in an  
Erlenmeyer flask at 37°C for 30 min with 60-90  
osc/min. The cells were then washed by centrifugation  
for 2 min, and the process repeated at least three  
times to eliminate peritubular cells. The, cells were  
30 resuspended by gentle homogenization with a Pasteur  
pipet in 40 mL media containing 40 mg hyaluronidase

1 and 0.2 mg of DNase, and incubated at 37°C for 30 min  
with 60-90 osc/min. The cells were pelleted by soft  
centrifugation for 2 min, and washed at least five  
times to eliminate germ cells. The resultant SEF was  
5 resuspended in 0.25 mL of media, and immediately  
transplanted into the recipient rat. Each grafted rat  
received the equivalent of the total amount of Sertoli  
cells contained in a single testis.

#### 10 Transplantation of Rats

The diabetic rat was anesthetized with  
methoxyflurane USP in a sterile hood and the left  
flank opened to expose the kidney. The Sertoli-  
enriched fraction containing approximately 5 million  
15 Sertoli cells was injected first underneath the renal  
capsule. The cells could be seen as a milkish bubble  
underneath the capsule. Immediately afterwards, a  
total of 10 islets/g of body weight was injected to  
the same milkish bubble. The needle was retracted  
20 slowly to prevent leakage of the grafted cells.  
Cyclosporine (CsA) was administered subcutaneously in  
varying doses over a 20-day period to groups two and  
four. Because the grafted rats responded similarly  
whether the drug was administered over a 20-day, or  
25 over a 3-day period, all of the subsequent groups,  
including the female rats, were treated with only  
three injections of 25 mg/kg CsA, given on days 0, +1,  
and +2, relative to the graft. The rats received no  
other therapy.

30 A total of 36 male and 21 female PVG rats  
were divided into six different treatment groups:

- 1 Group 1, the control group, consisted of 6 male rats  
grafted with only islets from S-D donor rats. They  
received neither SEF nor CsA. Group 2 consisted of 10  
rats grafted with a combination of islets from S-D  
5 rats and CsA postransplantation, but no SEF. Group 3  
consisted of a total of 10 rats grafted with a  
combination of islets from S-D and SEF from PVG donor  
rats, but no CsA postransplantation. Group 4  
consisted of 10 rats grafted with a combination of  
10 islets from S-D donors, SEF from PVG donors, and CsA  
postransplantation. Group 5 consisted of 11 female  
rats grafted with the same combination of cells as  
depicted for Group four. Group 6 consisted of 10  
female rats grafted with a combination of islets and  
15 SEF, both cell types from S-D donors, and CsA  
postransplantation.

#### Posttransplantation Evaluation of Rats

- The grafted rats were transferred to  
20 metabolic cages, and plasma glucose levels were  
obtained at weekly intervals. Urine volumes and urine  
glucose contents were obtained at daily intervals. A  
rat was considered cured of the diabetic process if  
the following criteria were met: A random plasma  
25 glucose level  $\leq 150$  mg/DL; glycosuria; and immediate  
reversal to hyperglycemia following surgical removal  
of the grafted kidney.

- To determine if any of the rats had become  
unresponsive to their grafts, normoglycemic rats were  
30 challenged with a secondary islet allograft consisting  
of at least 500, freshly prepared, Sprague Dawley

1 islets which were injected into the contralateral  
renal subcapsular space. No immunosuppression was  
given following the challenge.

To examine the impact of the transplantation  
5 of SEF on fertility of the female rats, normoglycemic  
animals of longer than 30 days were mated with PVG  
males. Metabolic parameters, as outlined above, were  
closely monitored, as was the course of their  
pregnancies.

10

#### Structural Analysis of Grafted Tissue

A total of five successfully grafted rats  
were nephrectomized at intervals following  
transplantation. Wedge sections of renal tissue,  
15 obtained from sites at which islets and SEF had been  
injected, were prepared for examination by light and  
electron microscopy, as previously described by  
Cameron et al. (1990) Transplantation, 50:649-653.  
Briefly, the tissue wedges were immersion-fixed with  
20 5% glutaraldehyde in 0.1 M collidine buffer for 1 h,  
washed in buffer, and postfixed for 1 h with 1% osmium  
tetroxide in 0.1 M buffer. Small tissue blocks were  
cut from the wedges, and dehydrated through a graded  
series of ethyl alcohols, transferred to propylene  
25 oxide, and embedded in Epon 812/Araldite plastic  
resin. Thick (0.5 $\mu$ m) and thin (900 mg) sections were  
stained routinely with toluidine blue and uranyl  
acetate/lead citrate, respectively, for structural  
analysis by light and electron microscopy. The  
30 results are shown in Table 3 and Figs. 7-9.

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TABLE 3

**Effect of Sertoli Cells on  
Islet Allograft Survival in the  
Non-Immunologically Privileged Renal, Subcapsular Site**

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Group (n)	Gender	Sertoli Cell (donor origin)	CsA	Duration of Normoglycemia (days) Individual Responses
1 (6)	Male	---	-	0,0,0,0,0,0
2 (10)	Male	---	+	0,0,0,0,0,0,130>441, >445
3 (10)	Male	+ (PVG)	-	0,0,0,0,9,10,12,13,13,14
4 (10)	Male	+ (PVG)	+	19,76,58*,84*,167*,127†, 139†,>418†,>422†,>425†
5 (11)	Female	+ (PVG)	+	7,11,14,28,>287†,>305†, >306†,>308†,>441†,>447†, >457†
6 (10)	Female	+ (S-D)	+	8,10,96*,128*,>168,>172, >184,>193,>193,>196

\* Nephrectomized

† Challenged with a Secondary Islet Allograft

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Group 1: None of the six rats grafted with islets alone, without either SEF or CsA, became normoglycemic.

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Group 2: Three of 10 rats grafted with islets and treated with CsA became normoglycemic for more than 100 days. The 3 normoglycemic rats were challenged with a secondary graft on days 116, 192 and 197, respectively. One rat reverted to hyperglycemia on day 130, while 2 remained normoglycemic.

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Group 3: Initially 6 of the 10 rats grafted with islets and SEF, but no CsA, became normoglycemic, but all of them reverted to hyperglycemia by day 14.

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1           Group 4: All 10 of rats grafted with a  
combination of SEF and islets, and also given CsA  
became normoglycemic. Two reverted spontaneously to  
diabetes on days 19 and 76, respectively. Three were  
5   nephrectomized on days 58, 84 and 167 following  
transplantation. All 3 of these rats became  
hyperglycemic within the next 24 h. The remaining 5  
rats were challenged with a secondary islet allograft  
on days 119, 129, 280 342 and 400, respectively. Of  
10 these, the first 2 reverted to diabetes on day 127 and  
139, respectively, while the latter 3 remained  
normoglycemic.

          Group 5: All 11 of the female rats grafted  
with a combination of islets and SEF, and then given  
15 CsA, became normoglycemic. of these, 4 reverted  
spontaneously to hyperglycemia by day 28. Of the 7  
normoglycemic rats who were mated with male PVG rats,  
6 became pregnant, and of these, 8 had litters varying  
between 1 and 10 pups. They were able to nurse the  
20 pups successfully. A total of 7 of the long-term  
surviving females were challenged with secondary islet  
allografts at least 200 days following  
transplantation. None of them reverted to  
hyperglycemia.

25           Group 6: of the 10 rats grafted with islets  
and SEF from the same donor strain of rat, all 10  
became normoglycemic. Two reverted to hyperglycemia  
by day 10. A nephrectomy to remove the graft was done  
on 2 of the long-term surviving rats on days 96 and  
30 201, respectively. Both reverted to hyperglycemic  
immediately within the next 24 h.

# 1 Tissue Morphology

Renal tissue obtained from the long-term grafted kidney appeared structurally normal by light microscopy (Figure 7). Transplanted islets in this organ were immediately subjacent to the kidney capsule, and also appeared structurally normal. They displayed tissue and cellular architecture identical to islets in situ (Figure 7). Individual islet cells were partitioned into cell clusters by thin connective septa containing small vessels and capillaries (Figure 7). It appeared that most of the islet cells contained secretion granules. When resolved by electron microscopy, islet cells were identified as the  $\beta$ -cell type by the inclusion of ultrastructurally distinctive, and unique insulin-containing secretion granules (Figure 8). All  $\beta$ -cell clusters observed were in close proximity to intra-islet capillaries (Figure 8).

There was a high density of cells between, and directly adjacent to, the transplanted islets and renal parenchyma. By light microscopy, they did not appear to be islet cells, kidney cells nor cells of blood origin (Figure 7). When observed by electron microscopy, these cells were similar in ultrastructure to Sertoli cells in that their nuclei were irregular in profile, and contained deep nuclear clefts, distinctive nucleoli were often present, and mitochondrial structure was dense. Although these cells did not retain the typical polarity of Sertoli cells in vivo, they were, however, identical in appearance to Sertoli cells in vitro, when the cells

- 1 are not plated on a basement membrane substrate. The  
cells were not associated with a basement membrane,  
and appeared randomly organized (Figure 9). Cells  
showing ultrastructural features of either germ or  
5 Leydig cells were not observed.

This example demonstrates that an  
immunologically privileged site for transplantation of  
isolated islet can be created in male and female  
diabetic recipients by transplantation of Sertoli  
10 cells without the need for sustained  
immunosuppression.

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EXAMPLE 4

This study determined the survival of discordant islet xenografts in various nonimmunologically privileged organ sites in experimental animals.

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Islets were prepared from young piglets as follows: Male piglets not weighing more than 2.2 kg were used exclusively. The piglet was anesthetized and following exsanguination both pancreas and testes were harvested under sterile conditions. A collagenase solution consisting of 2 mg/ml of collagenase type XI (Sigma) was injected directly into the pancreas. The pancreas was incubated at 37°C for 17 minutes and the digested tissues washed three times by means of centrifugation and aliquots of 1 ml each transferred to Petri dishes. The islets were incubated at 32°C in tissue culture media 199 supplemented with 10% horse serum for six days.

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On day seven the cultured islets were collected in batches of  $\pm 4,000$  and cryopreserved using a standard protocol. The cells were stored in liquid nitrogen at -96°C for periods varying between two and four weeks. The islets were removed from the liquid nitrogen and thawed using an established procedure. The thawed islets were transferred to Petri dishes and co-cultured with pig Sertoli cells for three days at 32°C in the same 199 culture media as described above. Earlier studies have shown an improved survival rate of thawed islets cultured in the presence of Sertoli cells.

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1           On day three following thawing the islets  
were hand-picked and counted and a total amount of 12  
islets/g of body weight transplanted into female  
diabetic Sprague Dawley rats. A total of 5 million  
5   Sertoli cells procured from the piglet testes were  
grafted simultaneously into the same location. The  
organ sites to be tested for the grafting of islets  
include: a) the renal subcapsular space, b)  
subcutaneously, and c) the liver. Following  
10   transplantation, the rats were treated with  
cyclosporine as follows: 25 mg/kg for 7 days; 15 mg/kg  
for 5 days; 10 mg/kg for 5 days; 5 mg/kg for an  
additional 13 days. On day 30 the drug was  
discontinued.

15           To demonstrate viability and functional  
integrity of isolated piglet islets the following  
studies were done: a) staining of Cells with  
dithizone, a stain is highly specific for insulin; b)  
staining of cells with 0.4% trypan blue which  
20   indicates viability of the islets; and c) culturing of  
batches of 5 islets in the presence of insulin  
secretagogues such as low and high glucose  
concentrations at specified intervals following  
culturing, cryopreservation and thawing. The results  
25   are shown in Table 4.

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**TABLE 4**

**Insulin Secretion (micro-units/ml) from  
Incubated and from Cryopreserved-Thawed Islets  
Done on Days 3, 7 and 14 of Culturing, Respectively**

5

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	3 DAYS	7 DAYS	14 DAYS
<b>INCUBATED ISLETS PRIOR TO CRYOPRESERVATION:</b>			
a) Low Glucose (90 mg/dl)	15.3±3.8	21.8±1.1	17.29±2.4
b) High Glucose (300 mg/dl)	32.2±5.4	37.14±3.4	23.3±1.8
<b>CRYOPRESERVED AND THAWED ISLETS:</b>			
a) Low Glucose (90 mg/dl)	14.52±2.8	7.13±1.3	5.38±2.02
b) Low Glucose + Sertoli Cells	10.31±2.8	9.17±2.6	8.38±.41

**TABLE 5**

**Yield of Porcine Islets  
Following 1, 3 and 7 Days of Culture and the  
Percentage of Islets Lost During 7 Days of Culture**

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Pig. No.	BW (kg)	Panc. W g	D1 Islets /g panc.	D3 Islets /g panc.	D7 Islets /g panc.	Islet Loss % D7/D1
1	1.6	1.79	36,536	31,659	27,212	26%
2	2.0	1.89	37,272	32,962	27,883	25%
3	2.3	2.46	29,268	26,046	20,884	29%
4	1.8	1.66	39,904	37,726	31,664	21%
5	1.8	1.76	37,846	34,578	30,046	21%
6	1.6	1.74	39,866	37,888	32,424	19%
7	1.4	1.61	42,126	39,456	33,872	20%
8	2.3	2.48	33,682	29,334	24,892	26%
9	2.1	2.28	43,478	41,226	37,394	14%
10	2.1	2.09	40,126	36,448	33,282	17%
11	2.1	2.12	31,248	27,170	26,415	15%
12	2.1	1.98	38,848	36,465	29,293	25%
13	2.2	2.06	39,146	37,446	31,709	19%
14	2.2	2.24	27,892	25,028	21,342	23%
15	2.7	2.69	44,610	38,364	31,524	29%
16	1.5	1.44	42,222	40,414	31,244	26%
Mean ± SE	2.0±0.3	2.0±0.4	37692±1233	34513±1307	29442±1119	22.2±1.2%

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TABLE 6

**Recovery of Islets Following Freezing and  
Thawing in Presence and Absence of Sertoli Cells**

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<u>Islets alone</u>				<u>Islets + Sertoli cells</u>				
	No. of islets	Pre-cryo		Recovery (%)	Pre-cryo		Post thawing	Recovery (%)
5	D3F/D3T	250	152	61%	290	212	73%	
		230	131	57%	260	228	88%	
		440	278	63%	430	280	88%	
		420	366	87%	410	324	79%	
		450	290	64%	440	358	81%	
10			Means	66.4%			81.8%	
	D7F/D3T	260	136	52%	250	229	92%	
		300	208	69%	300	202	67%	
		280	177	53%	290	238	82%	
		360	205	57%	350	300	86%	
15		320	218	68%	390	289	74%	
		380	217	57%	320	270	84%	
			Means	61.0%			80.8%	

20

As shown in Table 5, the yield of islets per gram pancreas was  $37692 \pm 1233$ ,  $34513 \pm 1307$  and  $29,442 \pm 1119$ , after 1, 3 and 7 days of culture, respectively. Following cryopreservation and thawing and reculturing of the cells in the presence of Sertoli cells approximately 20% of the cells were damaged or lost as shown in Table 6. Thus  $\pm 24,000$  islets/gram of piglet pancreas were available for transplant purposes after cryopreservation and thawing.

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1           The results showed that insulin secretion  
was blunted when glucose was used as insulin  
secretagogue prior to cryopreservation. The effect  
5           was more evident following cryopreservation and  
thawing. While the presence of Sertoli cells had  
marked effects on number of islets that survived  
cryopreservation and thawing their presence had little  
effect on the ability of the islets to respond to a  
low glucose concentration as insulin releasing agent.  
10          However, as shown in Example 8 the presence of Sertoli  
cells augmented the secretion of insulin in the  
presence of high glucose concentrations and glucose  
plus Forskolin.

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EXAMPLE 5

Response of Diabetic Sprague  
Dawley Rats to the Transplantation of  
Islets from Piglet Donors (Discordant Xenografts)

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The rats were made diabetic by means of a single i.v. injection of 55 mg/kg of streptozotocin. They were grafted only if the blood sugar was equal to or more than 400 mg/dl. Following transplantation the rats were placed individually in metabolic cages and urine volume, urine glucose content, and body weights were measured at daily intervals. Blood glucose levels were done at weekly intervals. A rat is considered cured of diabetes if the blood glucose level is 160 mg/dl or less and/or the daily urine volume is 15 ml or less.

10

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The results are illustrated in Figures 10 and 11.

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Figure 10 shows the effect of transplantation of piglet islets and Sertoli cells underneath the renal capsule on the mean daily urine output of seven grafted female rat recipients. Each bar represents the mean daily urine output over a ten-day period following transplantation. The study has been conducted over an 80-day period, the bar on the furthest right thus showing the mean urine output per day from day 80 through 89, etc.

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The figure shows that the mean daily urine volume for the first 60 days varied between 19.7 mls and 27 mls or within a diabetic range. It can be readily appreciated that urine volumes decreased to

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1 near-normal levels only from days 70 through day 89.  
The corresponding plasma glucose levels during the  
first and last ten day periods were  $474 \pm 46$  and  $155 \pm$   
70, mg/dl, respectively.

5 These results indicate that following  
transplantation with piglet islets and Sertoli cells  
the rats showed evidence of survival of the grafted  
islets. The reversal to normoglycemia took about 80  
days.

10 It should be noted that one of the cured  
rats is pregnant and has been normoglycemic throughout  
her pregnancy.

Figure 11 shows the effect of the  
transplantation of piglet islets and Sertoli cells  
15 underneath the skin on the mean daily urine volumes of  
three rats over a 50 day period. The results show  
that the mean urine volume decreased from a mean of  
41.7 ml during the first 10-day period to an average  
of 12.3 mls during the fifth week. The corresponding  
20 glucose levels were  $509 \pm 45$ , and  $200 \pm 12$ , mg/dl,  
respectively.

The data depicted above demonstrate that  
both the renal subcapsular space and the subcutaneous  
area can be used as a site to create an  
25 immunologically privileged site for the  
transplantation of islet xenografts.

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EXAMPLE 6

5 This study determined the effect of cultured Sertoli cells on the survival of discordant islet xenografts in diabetic rats with minimal early exogenous immunosuppression.

Preparation of Islets

10 Neonatal piglets of less than seven days of age were killed by anesthesia and islets were isolated according to a method of Kuo C.Y., Burghen G.A., Myvacle A. and Herrod H.G. (1994) "Isolation of islets from neonatal pig pancreatic tissue", J. Tissue Culture Methods, 16: 1-7. Briefly, the pancreas was  
15 distended by an injection of a collagenase solution, 2 mg/ml, collagenase type X1, in culture medium DMEM. After incubation at 39°C for 17 min, the digested fragments were washed by centrifugation and the digested tissue was then incubated for one week in  
20 medium 199 supplemented with 10% horse serum and 1% antibiotics at 32°C. The islets were then cryopreserved according to the method by Lakey J.R.T., Warnock G.L., Kneteman N.M., Ao Z., Rajotte R.V. (1994) "Effects of precryopreservation culture on  
25 human islet recovery and in vitro function", Transplant Proc., 26:820 and stored in liquid nitrogen at -196°C. Three days prior to transplantation the cryopreserved islets were rapidly thawed and cultured at 32°C for two days. One day prior to  
30 transplantation some of the islets were collected and co-cultured with Sertoli cells for 24 hours.

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# 1 Sertoli cell isolation

Testes of young S-D rats were removed and Sertoli cells were isolated by the method of Cheng C.Y. and Bardin C.W. (1987) "Identification of two testosterone responsive proteins in Sertoli cell-enriched culture medium whose secretion is suppressed by cells of the intact seminiferous tubule." J. Biol. Chem., 262:12768-12779. Briefly, the testes were digested first in DMEM containing 1.0% trypsin, and then in DMEM containing 1.0% collagenase, type 1, for periods of 15 min each, at 37°C. The purified Sertoli cells were cultured at 37°C in DMEM/F12 supplemented with transferrin, 10 ug/ml, FSH 10 ng/ml, insulin 20 ug/ml and 1.0% FCS, for three days. For transplantation, Sertoli cells and islets were pooled and rats were grafted with either a composite consisting of  $5 \times 10^6$  Sertoli cells and 3,000 islets, or with islets alone (15 islets/g of body weight).

## 20 Transplantation of rats

Female S-D rats, weighing between 170 and 200 g were made diabetic by means of a single i.v. injection of 60 mg/kg of streptozotocin. A total of 31 diabetic rats were divided into 3 groups and grafted as follows: Group 1, a control group (n=8), received a total of 15 islets/g body weight injected underneath the renal capsule. No Sertoli cells were grafted. Following transplantation the rats were treated with cyclosporine for 55 days: 25 mg/kg for 3 days, 15 mg/kg for 10 days, 10 mg/kg for 10 days and 5 mg/kg for the following 32 days. Immunosuppression

1 was then stopped. Each rat received, in addition, 1-3  
U of Ultralente insulin at daily intervals if the 24-  
hour urine glucose content exceeded 1 g. Insulin  
therapy was stopped on day 55. Group 1, a tissue  
5 control group (n=8), was given a renal, subcapsular  
injection of a composite of about  $5 \times 10^6$  Sertoli  
cells and 3,000 islets. No CsA was given. Insulin  
was given as depicted above. Group 3, the  
experimental group (n=15), was transplanted with both  
10 sertoli cells and islets and then treated with CsA and  
insulin according to the schedule outlined above.

#### Posttransplantation evaluation of rats

Plasma glucose levels were obtained at  
15 weekly intervals. Twenty four hour urine volumes and  
urine glucose contents were recorded daily. A rat was  
considered cured of the diabetic process if the  
following criteria applied: A plasma glucose level of  
equal to or less than 10 mmol/L, a 24-hour urine  
20 volume of less than 15 ml, and immediate reversal to  
hyperglycemia following surgical removal of the  
grafted kidney. One normoglycemic rat was mated on day  
69 to test her ability to become pregnant.

#### 25 Structural analysis of the grafted tissue

Two normoglycemic rats were nephrectomized  
on days 117 and 330 and grafted tissue prepared for  
light and electron microscopy. Selawry H.P., Cameron  
D.F. (1992) "Sertoli cell-enriched fractions in  
30 successful islet cell-transplantation", Cell Trans.,  
2:123-129. Briefly, tissue wedges were immersion-

- 1 fixed with 5% glutaraldehyde in 0.1 M collidine buffer  
for 1 h., washed in buffer, and postfixed for 1 h with  
1% osmium tetroxide in 0.1 M buffer. Small tissue  
blocks were cut from the wedges, and dehydrated  
5 through a graded series of ethyl alcohols, transferred  
to propylene oxide, and embedded in Epon 812/Araldite  
plastic resin. Thick (0.5 um) and thin (900 ng)  
sections were stained routinely with toluidine blue  
and urinal acetate/lead citrate, respectively, for  
10 structural analysis by light and electron microscopy.

The results of the effect of Sertoli cells  
and cyclosporine on survival of xenographic  
transplantation of pig islet cells into the renal  
subcapsular space of diabetic female rats are shown in  
15 Table 7.

**TABLE 7**

Group (n)	Sertoli Cells	CsA	Graft Survival (days)
1 (8)	-	=	0,0,0,0,0,0,0,0
2 (8)	+	-	0,0,0,0,0,0,0,0
3 (15)	+	+	0,0,0,0,0, 71, 77, 96, 117* 148#, >154, >165, >327, 330*

\* rats nephrectomized to remove the xenograft  
25 # rat died during a cardiac puncture

As shown in Table 7, none of the rats  
grafted with islets alone and then given CsA and low-  
dose insulin (Group 1) became significantly less  
30 hyperglycemic. Further, none of the rats grafted with  
a composite of islets and Sertoli cells, but without

1 CsA, showed any improvement of hyperglycemia (Group  
2). Of 15 rats grafted with islets and Sertoli cells  
and then given CsA (Group 3), 10 showed evidence of  
reversal of the diabetic state. Four of the ten are  
5 still normoglycemic for periods of more than 154, 165,  
165, and 327 days, respectively. The normoglycemic  
rats who were nephrectomized on days 117 and 330,  
became hyperglycemic immediately. Their plasma  
glucose levels were 4.9 mmol/L, and 8.2 mmol/L, prior  
10 to, and 20.7 mmol/L, and 32.2 mmol/L, respectively,  
following nephrectomy. A female rat who was mated on  
day 69 became pregnant and delivered a total of 10  
pups on day 89, all of whom she nursed successfully  
while remaining normoglycemic. She died on day 148 as  
15 a result of a cardiac puncture. Three of 10 rats  
regressed into hyperglycemia on days 71, 77, and 96,  
respectively, after a short period of euglycemia.

These results demonstrate that prolonged  
survival of a discordant islet xenograft (pig to rat)  
20 can be achieved in female diabetic rats. Survival of  
islet xenografts depended upon two factors which had  
to be administered concomitantly: Co-transplantation  
with Sertoli cells and treatment with cyclosporine.

The response of total urine volumes  
25 following transplantation with a composite of pig  
islet and rat Sertoli cells measured at 10-day  
intervals over an 80 day period for 7 of the improved  
rats showed an average daily urine volume of  $27.0 \pm$   
 $13.0$  ml/rat during the first 10-day period, which  
30 slowly declined to a mean of  $12.0 \pm 4.0$  ml/rat, 70  
days following transplantation.

1           Tissue morphology studies shown in Figure 12  
show that the tissue and cellular structure of kidney  
parenchyma appeared normal in the rat nephrectomized  
117 days following transplantation. Normal appearing  
5   islets with structurally distinct B-cells were visible  
in well vascularized areas subjacent to the kidney  
capsule. Additionally, normal appearing Sertoli cells  
were observed adjacent to the transplanted islets  
along with numerous lymphocytes. No plasma cells were  
10 identified at the transplantation site. Viable  
endocrine cells were similarly observed in the  
subcapsular renal space of the rat nephrectomized 330  
days following transplantation.

          These studies show that significant  
15 prolongation of survival of a discordant islet  
xenograft can be achieved without sustained  
immunosuppression. These studies demonstrate that the  
mechanism by which Sertoli cells promote islet  
xenograft survival is three-fold: (1) Sertoli cells  
20 stimulate the recovery of islets damaged during  
transplantation (i.e. improve the yield and function  
of cultured islets), (2) Sertoli cells protect grafted  
islets from immunologic rejection by producing factors  
which strongly suppress proliferation of T-cells, and  
25 (3) Sertoli cells protect grafted islets from the  
toxic effects of cyclosporine.

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EXAMPLE 7

5 This study shows a method of isolating and  
cryopreserving porcine pancreatic islets for future  
xenographic transplants in mammals.

Male piglets, < 7 days old and weighing 2±  
kg were used as donors. The pancreases, weighing  
1.4 ± 0.3 g, were harvested and injected with DMEM  
solution containing 2 mg/ml collagenase XI. The  
10 distended pancreas was incubated in a shaking water  
bath at 39°C for 17 min. The digested tissue was  
filtered through a 500 µm stainless steel filter and  
filtrates were washed x 3 with cold DMEM. Without  
further purification the cells were cultured in M199  
15 and 10% horse serum at 32°C for 7 days. The islet  
cells were then cryopreserved using standard  
procedures. At specified intervals islets were thawed  
and cultured in M199, both in presence, and isolated  
from testes of male piglets according to a standard  
20 method.

To test functional capacity, islets cultured  
for 3 and 7 days were assessed for insulin release in  
static incubation. In separate experiments, effect of  
insulin secretagogues was tested on islets cultured  
25 with and without Sertoli cells. The results of this  
study are shown in Tables 8 and 9.

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**TABLE 8**

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Effect of Insulin Secretagogues,  
Glucose and Glucose Plus Forskolin,  
on Insulin Release From Incubated and  
Frozen/Thawed (F/T) Islets in the Presence  
and Absence of Pig Sertoli Cells

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	Insulin Release (uU/ml/10islets)		
	3.3mmol/L glucose	16.7 mmol/L glucose	16.7 mmol/L glucose +100 umol Forskolin
Day 3 Incubated with Sertoli cells	42.3 ± 1.2	112.8 ± 17.7*#	267.7 ± 43.0**#
Day 3 Incubated alone	31.3 ± 2.1	57.3 ± 3.8*	123.4 ± 15.3 **
Day 7 Incubated with Sertoli cells	22.9 ± 1.9	64.5 ± 6.4*#	153.9 ± 14.6**
Day 7 Incubated alone	21.3 ± 1.2	37.3 ± 6.0*	120.3 ± 11.4**
Day 3 F/T with Sertoli cells	20.6 ± 4.3	44.9 ± 9.9*	77.1 ± 13.7**
Day 3 F/T alone	11.7 ± 2.3	27.9 ± 6.6*	54.5 ± 10.7**

Anova Test: \* vs 3.3mmol/L p 0.05, \*\* vs both 3.3 & 16.7 mmol/L P<0.05  
# with Sertoli cells vs islets alone P<0.05

**TABLE 9**

Effect of Sertoli cells on insulin content of incubated and  
frozen-thawed piglet islets.

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Insulin content (uU/10 islet(s))		
	Islets alone	Islets & Sertoli cells
Incubated D1	257.0 ± 19.6	391.1 ± 51.4*
Incubated D3	201.1 ± 19.1#	400.1 ± 41.0*#
Incubated D7	179.1 ± 26.2#	271.9 ± 39.9*#
Frozen D3/Thaw D3	52.4 ± 10.3	132.5 ± 35.1
Frozen D7/Thaw D3	10.4 ± 0.9	35.1 ± 8.2

Anova \* islets + Sertoli cell vs. islet alone P<0.05

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1           # Incubated islets D3, D7 vs. Frozen D3, D7 P<0.05

          These results show that: (1) large numbers  
of neonatal porcine islets can be isolated by a simple  
method; (2) cryopreservation and thawing results in  
5       about 40% loss in number of islets in the absence of  
Sertoli cells and about a 20% loss in the presence of  
Sertoli cells ; (3) cultured islets have the ability  
to respond to both glucose and glucose + Forskolin as  
insulin secretagogues; (4) the functional capacity of  
10       the cocultured islet was enhanced two-fold in the  
presence of Sertoli cells; (5) following  
cryopreservation and thawing, islets recover more  
rapidly in presence of Sertoli cells and the response  
to both glucose and glucose + Forskolin was enhanced  
15       two fold in the presence of Sertoli cells.

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EXAMPLE 8

This example describes a method of treating genetic diabetes which is demonstrated using the animal model NOD (non-Obese Diabetic).

Genetic diabetic mice (NOD) are recipients of pancreatic islet xenografts. Diabetic mice are selected from the colony of NOD mice maintained at the University of Tennessee Medical Center. The current incidence of diabetes in this colony is 80% for females and 63% of males by 25 weeks of age. Animals are considered diabetic if they have two consecutive weekly urine glucose readings of  $\geq 3+$  and a confirmatory plasma glucose greater than 400 mg/dl. Three to ten days before transplantation of the graft, the animals receive appropriate insulin to stabilize their health. Diabetic animals are randomly divided into two groups. All animals in these two groups receive 0.3 mg of the antiCD4 antibody, GK1.5 on days -1, 0 and 1 to initiate immunosuppression. Maintenance immunosuppression with GK1.5 cyclosporine A, FK506, cyclophosphamide, rapamycin, nicotinamide or 15-deoxyspergualin may be required.

Porcine pancreatic islets for transplantation are prepared as described in Example 6 except that they are not cryopreserved. Nicotinamide (10mM) or IGF-1 may be added to the incubation medium prior to transplantation. Porcine sertoli cells for transplant are prepared as described in Example 6. Cyclosporine may be included in the culture medium

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- 1 during the first four incubation days prior to  
transplantation.

One group of the diabetic NOD mice receive  
3,000 porcine islets in 25 $\mu$ l of Hank's buffered salt  
5 solution (HBSS) under the right renal capsule followed  
by an injection of  $2 \times 10^7$  pig Sertoli cells in 25 $\mu$ l  
in HBSS under the same renal capsule. A second group  
of mice receive a transplant consisting of only the  
porcine islet cells. Animals with xenografts continue  
10 to receive daily insulin injections; the amount  
determined by the concentration of glucose in the  
animals urine and plasma. Mice with plasma glucose  
levels less than 250 mg/dl are considered cured and no  
additional insulin administered.

15 A majority of mice receiving porcine sertoli  
cells and porcine pancreatic islets attain normal  
urine and plasma glucose levels (xenograft acceptors).  
In contrast, the majority of mice receiving porcine  
islets alone exhibit graft failure and do not attain  
20 normal glucose levels in the urine or plasma.

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EXAMPLE 9

5 This example shows a method of monitoring the immune response elicited against cellular transplants.

10 Total spleen leukocytes are isolated from mice by methods known in the art from mice that have rejected their porcine islet xenografts. In preliminary experiments these leukocytes are administered to NOD/scid mice (NOD mice that in addition have genetic immunodeficiency) with successful pig islet xenografts. The leukocytes cause rejection of the xenograft.

15 Plastic adherence (to deplete macrophages), nylon wool adherence (to deplete non-T lymphocytes), or specific antibodies (anti-CD4, anti-CD8, anti-F4/80, anti-B220) are used to deplete the total splenocyte preparations of certain classes of leukocytes. The class-depleted leukocyte preparations are injected into the NOD/scid mice with successful pig islet xenografts to determine which leukocyte class is necessary and sufficient to cause xenograft rejection.

25 Spleen leukocytes are isolated by conventional methods known in the art from mice (xenograft acceptor of Example 8) that have accepted their pig islet xenografts. These leukocytes are administered to NOD/scid mice with successful pig islet xenografts. The leukocytes do not cause rejection of the xenograft. Combining the appropriate leukocyte-depleted preparation that causes rejection

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- 1 with leukocytes from a xenograft acceptor of Example 8  
(50/50 mixture) and administering the mixed cell  
population to NOD/scid xenograft recipients (adoptive  
transfer) allows determination of whether so-called  
5 suppressor lymphocytes are preventing xenograft  
rejection in xenograft acceptors.

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EXAMPLE 10

5 This example provides a method of encapsulation of Sertoli cells with islets for transplantation.

**Preparation of Islets and Sertoli Cell Isolation**

10 Islets are isolated from female Fischer rats and are incubated in CMRL medium for approximately four days prior to usage. Sertoli cells are isolated from weanling male Fischer rats and are incubated for approximately four days to confluency in Petri dishes at 37°C.

15 After four days of incubation, the islets are counted and groups of 50 islets are transferred to 24 well Petri dishes. Sertoli cells are removed from Petri dishes with Sigma non-enzymatic media. The Sertoli cells are washed and counted.

20 Three experimental groups are then established as follows: Group 1: 12-well Petri dishes, each well containing 50 islets; Group 2: 12-well Petri dishes, each well containing a total of  $1 \times 10^4$  Sertoli cells; and Group 3: 12-well Petri dishes, each well containing 50 islets, plus  $1 \times 10^4$  Sertoli  
25 cells. The Petri dishes are incubated at 37°C for 24 hours to permit Sertoli cells to attach to islets.

**Microencapsulation of Islets and Sertoli Cells**

30 Islets, Sertoli cells and islets plus Sertoli cells are encapsulated by suspension in a solution of sodium alginate which is sprayed into a

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- 1 dish of calcium chloride using a droplet forming device according to the method of Lim et al. (1980 Science 210:908-910, the contents of which is incorporated by reference. The droplets are coated
- 5 with a layer of poly-L-lysine (PLL) with an average size of 20 kDa at a concentration of 0.05% (w/v) and a reaction time of 6 minutes according to the method of Goosen et al. (1985) Biotechnol. Bioeng. 27:146-150, the contents of which is incorporated by reference.
- 10 An additional outer layer of sodium alginate is added around the capsule according to the method of O'Shea et al. (1984) Biochim. Biophys. Acta 804:133-136, incorporated herein by reference. Alternatively, isolated cells may be encapsulated according to the
- 15 methodology of Weber et al. U.S. Patent No. 5,227,298, incorporated herein by reference. Following encapsulation, the cells are divided into treatment groups. Group 1: Free islets, not encapsulated, Group 2: Islets alone, encapsulated, Group 3: Sertoli cells
- 20 alone, encapsulated, and Group 4: Islets plus Sertoli cells encapsulated. Encapsulated cells are placed in media conventionally selected by the skilled artisan at 37°C.

25 **In Vitro Encapsulation**

At specified intervals following encapsulation, i.e., 1, 7, 14, 21 and 30 days respectively, following incubation, functionality of the groups containing islets are examined.

- 30 Approximately 10 capsules from each of the islet containing groups are stimulated, in tandem, by a

- 1 buffered medium containing glucose 9 mmol/L, glucose  
at 16.7 mmol/L, and Forskolin at 10mM for 30 minutes,  
in a water bath at 37°C. The perfusate is collected  
and insulin is assayed using a commercially available  
5 kit (e.g., Linco insulin kit). Insulin content of  
free encapsulated islets and islets encapsulated with  
Sertoli cells is further examined via an acid-ethanol  
extract of said capsules and assayed for insulin  
content using a commercially available kit (e.g. Linco  
10 insulin kit).

#### In Vivo Encapsulation

- Female Wistar-Furth rats are made diabetic  
by means of a single i.v. injection of streptozotocin.  
15 A total of 32 diabetic rats are divided into four  
groups and treated as follows: Group 1, a control  
group (n=8), will receive an intraperitoneal injection  
of at least 10 capsules containing Sertoli cells  
alone; Group 2 (n=8) will receive an intraperitoneal  
20 injection of 10 islets/g of free, non-encapsulated  
islets; Group 3 (n=8) will receive an i.p. injection  
of 10 islets/g of encapsulated islets alone; Group 4,  
(n=8) will receive 10 islets/g of co-encapsulated  
islets plus Sertoli cells. No group will receive any  
25 immunosuppression following transplantation. All rats  
are closely monitored via daily plasma glucose levels  
for the first week post-transplantation, and then at  
weekly intervals thereafter.

- Rats are considered cured of diabetes if  
30 they exhibit a blood glucose level less than or equal

1 to 170 mg/dl with concomitant steady increases in body weight.

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This example describes a method of immortalizing Sertoli cells using a temperature-sensitive mutant of the SV40 virus.

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Sertoli cells are isolated from sexually mature (120 days) rats according to the method of Wright et al. (1989) Ann. NY Acad. Sci. 564:173-185. The testes are removed and the tubules are resuspended in DMEM F-12 (DMEM/F12: Life Technologies, Inc., Grand Island, NY) containing 1mg/ml collagenase (Worthington, Freehold, NJ), 2mg/ml hyaluronidase (Sigma, St. Louis, MO), 0.3 mg/ml DNase (Sigma), and 65ug/ml soybean trypsin inhibitor (Sigma), and are incubated for 25 min at 32°C with gentle shaking. The incubation is repeated and the tubules are washed in F12/DMEM and digested in an enzyme solution including 1mg/ml collagenase/dipase (Boehringer-Mannheim, Indianapolis, IN) in lieu of collagenase. The tubules are recovered and further broken up by gentle pipetting with a Pasteur pipette. The digestion mixture is filtered through a nylon mesh to remove clumps of undispersed Sertoli cells. The Sertoli cells are then sedimented at unit gravity yielding a 95% pure population of adult Sertoli cells.

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Two 25-cm<sup>2</sup> flasks are seeded with Sertoli cells at a density of  $5 \times 10^6$  cells per plate. These flasks are incubated with SV40 virus mutant tsA255 for 3 h. The virus-containing medium is removed, and the cells are incubated at 33°C in F12/DMEM supplemented with 4% fetal bovine serum (FBS). Foci of transformed cells are visible at 6 weeks after infection. Each

1 individual focus is isolated with sterile metal rings,  
the cells are isolated from the plate with trypsin  
EDTA, and the cell suspensions are replated in 25-cm<sup>2</sup>  
flasks. Aliquots of cells from each focus are  
5 cultured at 33°C or 40°C for two days. At the end of  
this culture period, the cells are collected and total  
RNA is isolated for Northern blot analysis according  
to the methodology of Roberts, et al. (1992) Biol.  
Reprod. 47:92-96, incorporated herein by reference.  
10 Sertoli cells are selected for cloning on the basis of  
the inducible expression of mRNAs encoding Sertoli  
cell-secreted proteins according to the methods of  
Roberts, et al. (1995) Biol. Reprod. 53:1446-1453,  
incorporated herein by reference. Clonal cells are  
15 cultured in DMEM/F12 Plus 4% FBS, supplemented with 1%  
antibiotic-antimycotic. The cells are then seeded and  
allowed to attach at 33°C for at least 24 hours.  
Clonal cells are collected by washing the plates twice  
with Hanks balanced salt solution followed by a brief  
20 incubation with trypsin/EDTA.

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1    WHAT IS CLAIMED IS:

1. A method of treating a disease that results from a deficiency of a biological factor in a mammal wherein said method comprises administering  
5    Sertoli cells and a therapeutically effective amount of cells that produce said biological factor to a mammal in need of such treatment, wherein said Sertoli cells are administered in an amount effective to create an immunologically privileged site.

10          2. The method of Claim 1 wherein said mammal is a human.

3. The method of Claim 1 wherein said biological factor is a hormone.

15          4. The method of Claim 1 wherein said biological factor is insulin and said disease is diabetes mellitus.

5. The method of Claim 4 wherein said cells that produce said biological factor are pancreatic islet of Langerhans cells.

20          6. The method of Claim 1 wherein said cells that produce said biological factor are cells transformed by a nucleic acid encoding said biological factor.

25          7. The method of Claim 1 wherein said administering is by transplantation.

8. The method of Claim 1 wherein said Sertoli cells are administered in a dosage ranging from  $10^5$  to  $10^{10}$  cells.

30          9. The method of Claim 1 wherein said cells that produce said biological factor are administered in a dosage of from  $10^5$  to  $10^{10}$  cells.

1           10. The method of Claim 7 wherein said  
transplantation is by xenograft.

          11. The method of Claim 7 wherein said  
transplantation is by allograft.

5           12. The method of Claim 1 which further  
comprises administering an immunosuppressive agent.

          13. The method of Claim 12 wherein said  
immunosuppressive agent is administered for a time  
sufficient to permit said transplanted cells to be  
10 functional.

          14. The method of Claim 12 wherein said  
immunosuppressive agent is cyclosporine.

          15. The method of Claim 14 wherein said  
cyclosporine is administered at a dosage of from 5 to  
15 40 mg/kg body wt.

          16. The method of Claim 1 which further  
comprises administering a therapeutically effective  
amount of exogenous biological factor following the  
transplantation of said cells that produce said  
20 biological factor.

          17. The method of Claim 1 wherein said  
cells that produce said biological factor are co-  
cultured with Sertoli cells in tissue culture.

          18. The method of Claim 17 wherein said  
25 cells that produce said biological factor are  
cryopreserved prior to co-culturing with Sertoli cells  
in tissue culture.

          19. The method of Claim 1 wherein said  
Sertoli cells are obtained from a cell line.

30           20. The method of Claim 19 wherein said  
Sertoli cells are obtained by the steps comprising:

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- 1           a. isolating mammalian Sertoli cells from  
mammalian tissue;
- b. incubating said isolated mammalian  
Sertoli cells with virus producing cells under
- 5       conditions sufficient to transform said Sertoli cells;
- c. isolating said transformed Sertoli cells  
from the virus producing cell; and
- d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for
- 10       cloning.
21. The method of Claim 19 wherein said  
Sertoli cells are obtained by the steps comprising:
  - a. isolating mammalian Sertoli cells from  
mammalian tissue;
  - 15       b. incubating said isolated mammalian  
Sertoli cells with a mutagen under conditions  
sufficient to transform said Sertoli cells;
  - c. collecting said transformed Sertoli  
cells; and
  - 20       d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.
22. The method of Claim 20, wherein said  
virus producing cells are SV40 or polyoma virus.
- 25       23. The method of Claim 1, wherein the  
Sertoli cells and the cells that produce a biological  
factor are co-localized.
24. The method of Claim 23, wherein the  
Sertoli cells and the cells that produce a biological
- 30       factor are co-encapsulated.

1           25. A method of treating diabetes mellitus  
in a mammal wherein said method comprises  
administering to a diabetic mammal Sertoli cells in an  
amount effective to create an immunologically  
5 privileged site and a therapeutically effective amount  
of pancreatic islet of Langerhans cells.

26. The method of Claim 25 wherein said  
diabetes mellitus is type I or type II.

10 27. The method of Claim 25 wherein said  
mammal is a human.

28. The method of Claim 25 wherein said  
Sertoli cells are human, bovine or porcine.

15 29. The method of Claim 25 wherein said  
pancreatic islet of Langerhans cells are human, bovine  
or porcine.

30. The method of Claim 25 wherein said  
administering is by transplantation.

20 31. The method of Claim 30 wherein said  
transplantation is by injection into the renal  
subcapsular space.

32. The method of Claim 30 wherein said  
transplantation is by injection into the subcutaneous  
facie.

25 33. The method of Claim 25 wherein said  
Sertoli cells are administered at a dosage ranging  
from  $10^5$  to  $10^{10}$  cells.

34. The method of Claim 25 wherein said  
islet of Langerhans cells are administered at a dosage  
ranging from 5-1000 islet cells/g body wt.

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1           35. The method of Claim 25 which further  
comprises the administration of an immunosuppressive  
agent.

5           36. The method of Claim 35 wherein said  
immunosuppressive agent is administered for a time  
sufficient to permit the transplanted islets to be  
functional.

          37. The method of Claim 35 wherein said  
immunosuppressive agent is cyclosporine.

10          38. The method of Claim 35 wherein said  
cyclosporine is administered at a dosage of 5 to 40  
mg/kg body wt.

          39. The method of Claim 25 which further  
comprises administering a therapeutically effective  
15 amount of insulin following transplantation of said  
pancreatic islet of Langerhans cells.

          40. The method of Claim 25 wherein said  
Sertoli cells are obtained from a cell line.

20          41. The method of Claim 40 wherein said  
Sertoli cells are obtained by the steps comprising:

          a. isolating mammalian Sertoli cells from  
mammalian tissue;

          b. incubating said isolated mammalian  
Sertoli cells with virus producing cells under  
25 conditions sufficient to transform said Sertoli cells;

          c. isolating said transformed Sertoli cells  
from the virus producing cell; and

          d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
30 cloning.

- 1                   42. The method of Claim 40 wherein said  
Sertoli cells are obtained by the steps comprising:
- 5                   a. isolating mammalian Sertoli cells from  
mammalian tissue;
- b. incubating said isolated mammalian  
Sertoli cells with a mutagen under conditions  
sufficient to transform said Sertoli cells;
- c. collecting said transformed Sertoli  
cells; and
- 10                  d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.
43. The method of Claim 41 wherein said  
virus producing cells are SV40 or polyoma virus.
- 15                  44. The method of Claim 25, wherein the  
Sertoli cells and the pancreatic islet of Langerhans  
cells are co-localized.
45. The method of Claim 25, wherein the  
Sertoli cells and the pancreatic islet of Langerhans  
20 cells are co-encapsulated.
46. A method of creating an immunologically  
privileged site in a mammal wherein said method  
comprises transplanting isolated Sertoli cells into a  
mammal.
- 25                  47. The method of Claim 46 wherein said  
mammal is a human.
48. The method of Claim 46 wherein said  
Sertoli cells are injected into the renal subcapsular  
space.

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1           49. The method of Claim 46 wherein said  
Sertoli cells are injected into the subcutaneous  
facie.

5           50. The method of Claim 46 wherein said  
Sertoli cells are transplanted at a dosage ranging  
from  $10^5$  to  $10^{10}$  cells.

51. The method of Claim 46 wherein said  
Sertoli-cells are human, bovine or porcine.

10          52. The method of Claim 46 wherein said  
Sertoli cells are obtained from a cell line.

53. The method of Claim 50 wherein said  
Sertoli cells are obtained by the steps comprising:

- a. isolating mammalian Sertoli cells from  
mammalian tissue;
- 15          b. incubating said isolated mammalian  
Sertoli cells with virus producing cells under  
conditions sufficient to transform said Sertoli cells;
- c. isolating said transformed Sertoli cells  
from the virus producing cell; and
- 20          d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.

54. The method of Claim 50 wherein said  
Sertoli cells are obtained by the steps comprising:

- 25          a. isolating mammalian Sertoli cells from  
mammalian tissue;
- b. incubating said isolated mammalian  
Sertoli cells with a mutagen under conditions  
sufficient to transform said Sertoli cells;
- 30          c. collecting said transformed Sertoli  
cells; and

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1           d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.

5           55. The method of Claim 53 wherein said  
virus producing cells are SV40 or polyoma virus.

10           56. A method of creating systemic tolerance  
to subsequent transplants comprising transplanting  
Sertoli cells prior to said subsequent transplant with  
a transplant in an amount sufficient to tolerize said  
mammal.

          57. The method of Claim 56 wherein said  
transplants are endocrine cells.

15           58. The method of Claim 56 wherein said  
Sertoli cells are administered in a dosage ranging  
from  $10^5$  to  $10^{10}$  cells.

          59. The method of Claim 56 wherein  
endocrine cells are simultaneously transplanted with  
said Sertoli cells.

20           60. The method of Claim 56 wherein said  
Sertoli cells are obtained from a cell line.

          61. The method of Claim 60 wherein said  
Sertoli cells are obtained by the steps comprising:

          a. isolating mammalian Sertoli cells from  
mammalian tissue;

25           b. incubating said isolated mammalian  
Sertoli cells with virus producing cells under  
conditions sufficient to transform said Sertoli cells;

          c. isolating said transformed Sertoli cells  
from the virus producing cell; and

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1           d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.

5           62. The method of Claim 50 wherein said  
Sertoli cells are obtained by the steps comprising:  
a. isolating mammalian Sertoli cells from  
mammalian tissue;

10          b. incubating said isolated mammalian  
Sertoli cells with a mutagen under conditions  
sufficient to transform said Sertoli cells;

        c. collecting said transformed Sertoli  
cells; and

15          d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.

        63. The method of Claim 61 wherein said  
virus producing cells are SV40 or polyoma virus.

20          64. A method of treating an autoimmune  
disease in a mammal wherein said method comprises  
administering to said mammal a therapeutically  
effective amount of Sertoli cells.

        65. The method of Claim 64 wherein said  
Sertoli cells are administered in a dosage ranging  
from  $10^5$  to  $10^{10}$  cells.

25          66. The method of Claim 64 wherein said  
Sertoli cells are obtained from a cell line.

        67. The method of Claim 64 wherein said  
Sertoli cells are obtained by the steps comprising:

30          a. isolating mammalian Sertoli cells from  
mammalian tissue;

- 1           b. incubating said isolated mammalian  
Sertoli cells with virus producing cells under  
conditions sufficient to transform said Sertoli cells;  
c. collecting said transformed Sertoli  
5 cells from the virus producing cell; and  
d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.

68. The method of Claim 64 wherein said  
10 Sertoli cells are obtained by the steps comprising:  
a. isolating mammalian Sertoli cells from  
mammalian tissue;  
b. incubating said isolated mammalian  
Sertoli cells with a mutagen under conditions  
15 sufficient to transform said Sertoli cells;  
c. isolating said transformed Sertoli  
cells; and  
d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
20 cloning.

69. The method of Claim 67 wherein said  
virus producing cells are SV40 or polyoma virus.

70. A method of enhancing the maturation,  
proliferation and functional capacity of mammalian  
25 cells in tissue culture comprising co-culturing said  
cells with Sertoli cells.

71. The method of Claim 70 wherein said  
Sertoli cells are co-cultured in an amount ranging  
from  $10^5$  to  $10^{10}$  cells.

- 30           72. The method of Claim 70 wherein said  
Sertoli cells are obtained from a cell line.

- 1           73. The method of Claim 72 wherein said  
Sertoli cells are obtained by the steps comprising:
- a. isolating mammalian Sertoli cells from  
mammalian tissue;
- 5           b. incubating said isolated mammalian  
Sertoli cells with virus producing cells under  
conditions sufficient to transform said Sertoli cells;
- c. isolating said transformed Sertoli cells  
from the virus producing cell; and
- 10          d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.

74. The method of Claim 72 wherein said  
Sertoli cells are obtained by the steps comprising:
- 15          a. isolating mammalian Sertoli cells from  
mammalian tissue;
- b. incubating said isolated mammalian  
Sertoli cells with a mutagen under conditions  
sufficient to transform said Sertoli cells;
- 20          c. collecting said transformed Sertoli  
cells; and
- d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.

- 25          75. The method of Claim 70 wherein said  
virus producing cells are SV40 or polyoma virus.

76. The method of Claim 70 wherein the  
mammalian cells and the Sertoli cells are co-  
localized.

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1           77. The method of Claim 70 wherein the  
mammalian cells and the Sertoli cells are co-encapsulated.

5           78. A method of enhancing the recovery rate  
and viability of frozen mammalian cells in tissue  
culture-comprising co-culturing said cells with  
Sertoli cells.

          79. The method of Claim 78 wherein said  
mammalian cells are endocrine cells or germ cells.

10          80. A method of enhancing the recovery and  
proliferation of ex vivo cells comprising co-culturing  
said cells with Sertoli cells for a time and under  
conditions sufficient to achieve said enhanced  
recovery and proliferation.

15          81. A method of enhancing the recovery and  
proliferation of ex vivo cells comprising culturing  
said cells with a culture media from a tissue culture  
containing Sertoli cells for a time and under  
conditions sufficient to achieve said enhanced  
recovery and proliferation.

20          82. A pharmaceutical composition comprising  
Sertoli cells and cells that produce a biological  
factor and a pharmaceutically acceptable carrier.

          83. The composition of Claim 82 wherein  
said biological factor is a hormone.

25          84. The composition of Claim 82 wherein  
said cells that produce a biological factor are  
pancreatic islet of Langerhans cells.

30          85. The composition of Claim 82 wherein  
said cells that produce said biological factor are  
cells that are transformed by a nucleic acid encoding  
said biological factor.

1           86. The composition of Claim 82 wherein  
said Sertoli cells are obtained from a cell line.

          87. The composition of Claim 82 wherein  
said Sertoli cells are obtained by the steps  
5 comprising:

- a. isolating mammalian Sertoli cells from  
mammalian tissue;
- b. incubating said isolated mammalian  
Sertoli cells with virus producing cells under  
10 conditions sufficient to transform said Sertoli cells;
- c. isolating said transformed Sertoli cells  
from the virus producing cell; and
- d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
15 cloning.

          88. The composition of Claim 82 wherein  
said Sertoli cells are obtained by the steps  
comprising:

- a. isolating mammalian Sertoli cells from  
20 mammalian tissue;
- b. incubating said isolated mammalian  
Sertoli cells with a mutagen under conditions  
sufficient to transform said Sertoli cells;
- c. collecting said transformed Sertoli  
25 cells; and
- d. optionally screening transformed Sertoli  
cells for expression of an appropriate isolate for  
cloning.

          89. The composition of Claim 82 wherein  
30 said virus producing cells are SV40 or polyoma virus.

          90. A pharmaceutical composition comprising

- 1 Sertoli cells, pancreatic islet of Langerhans cells  
and a pharmaceutically acceptable carrier.

91. A pharmaceutical composition comprising  
Sertoli-cells and a pharmaceutically acceptable  
5 carrier.

92. A compartmentalized kit adapted to  
receive a first container adapted to contain Sertoli  
cells and a second container adapted to contain cells  
that produce a biological factor that is absent or  
10 defective in a disease.

93. A compartmentalized kit adapted to  
receive a first container adapted to contain Sertoli  
cells and a second container adapted to contain  
pancreatic islet of Langerhans cells.

94. An article of manufacture comprising a  
packaging material and Sertoli cells contained within  
said packaging material, wherein said Sertoli cells  
are effective for creating an immunologically  
privileged site in a mammal, and wherein said  
20 packaging material contains a label that indicates  
that said Sertoli cells can be used for creating an  
immunologically privileged site in a mammal.

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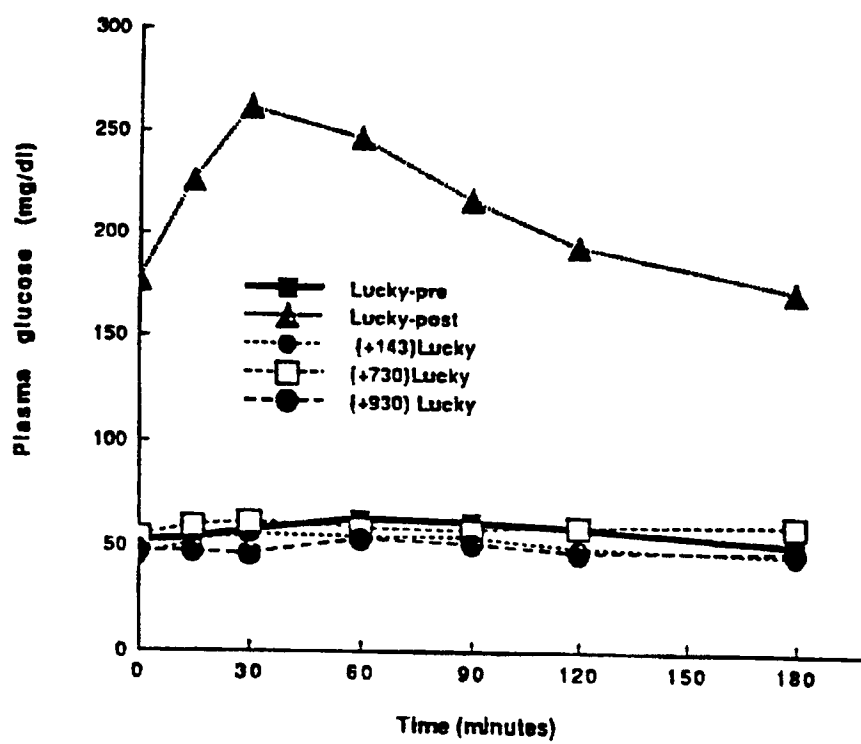


FIGURE 1

SUBSTITUTE SHEET (RULE 26)

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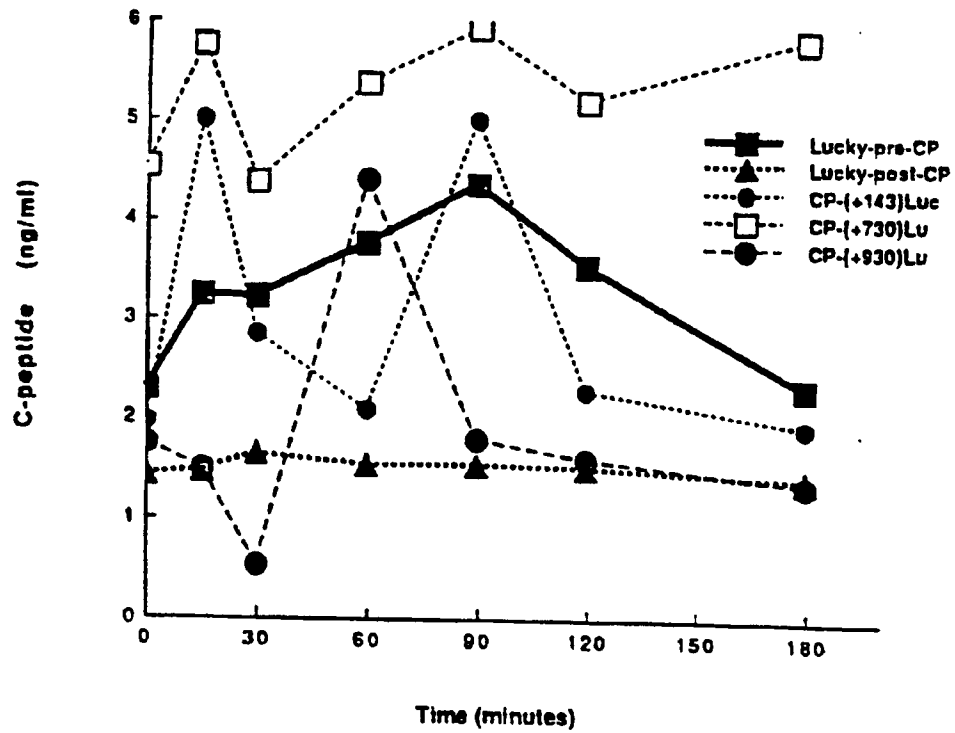


FIGURE 2

SUBSTITUTE SHEET (RULE 26)

3 / 1 2

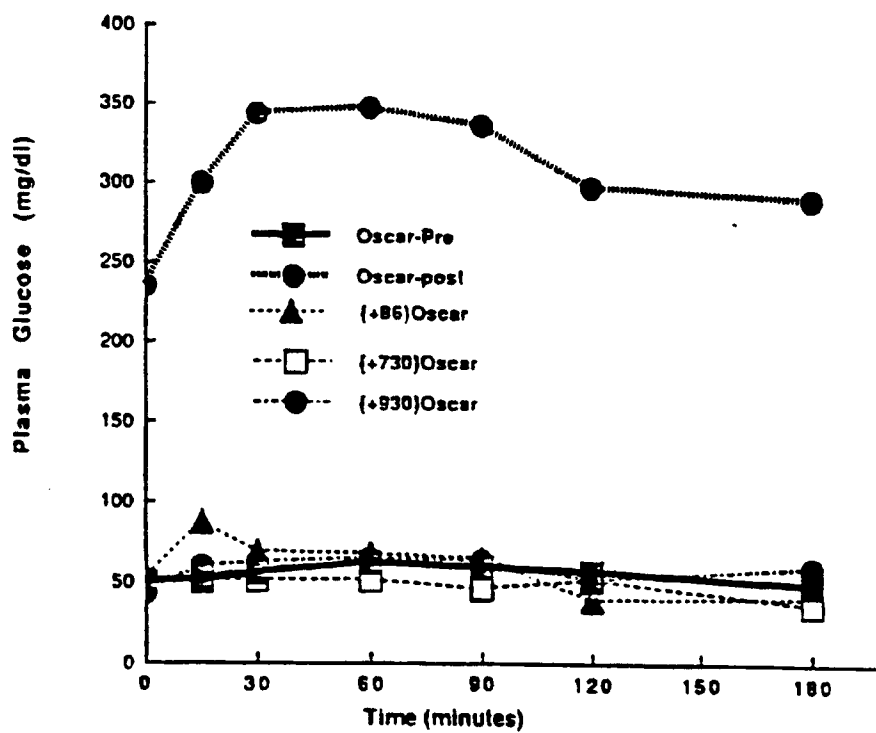


FIGURE 3

SUBSTITUTE SHEET (RULE 26)

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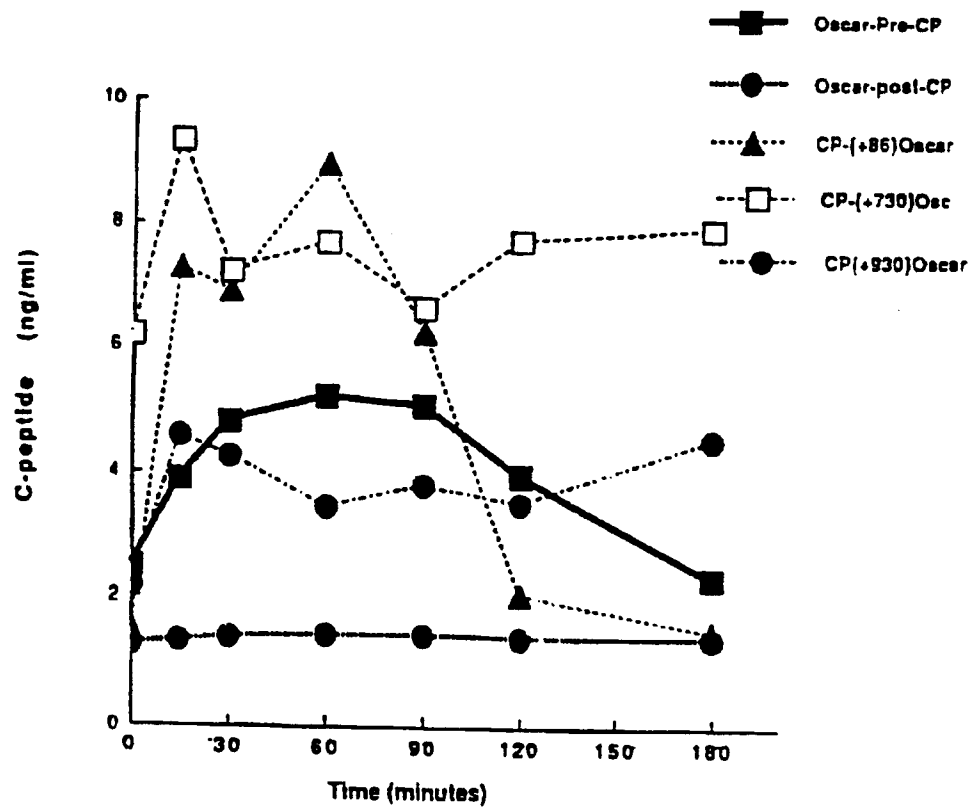
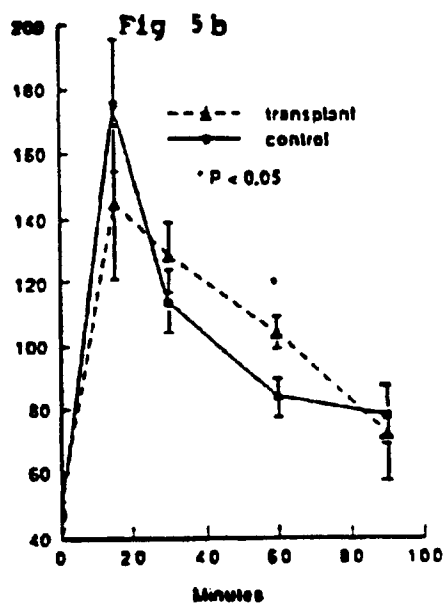
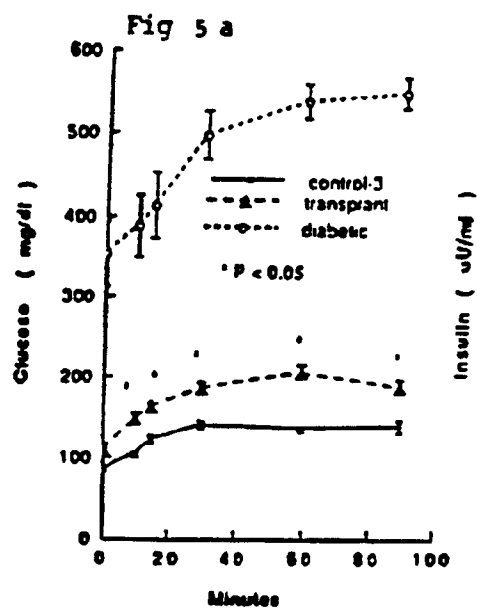


FIGURE 4

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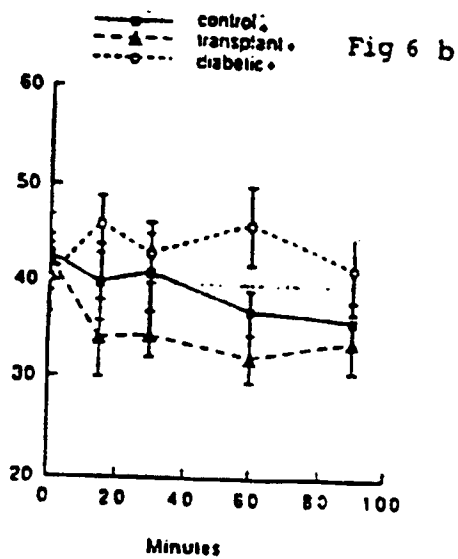
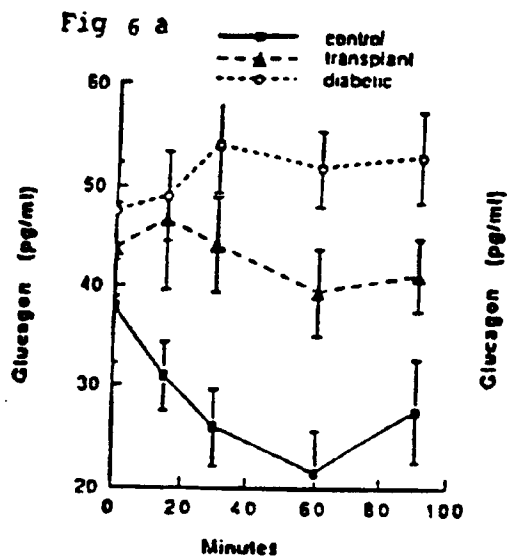
5 / 1 2



FIGURES 5a and 5b

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FIGURES 6a and 6b

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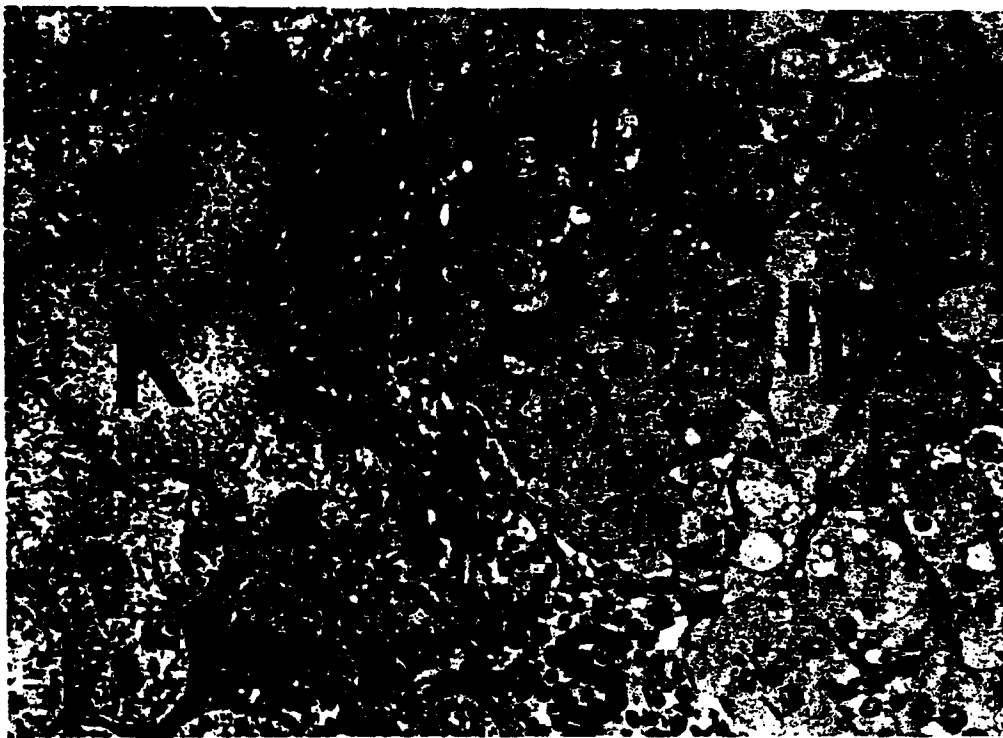


FIG. 7

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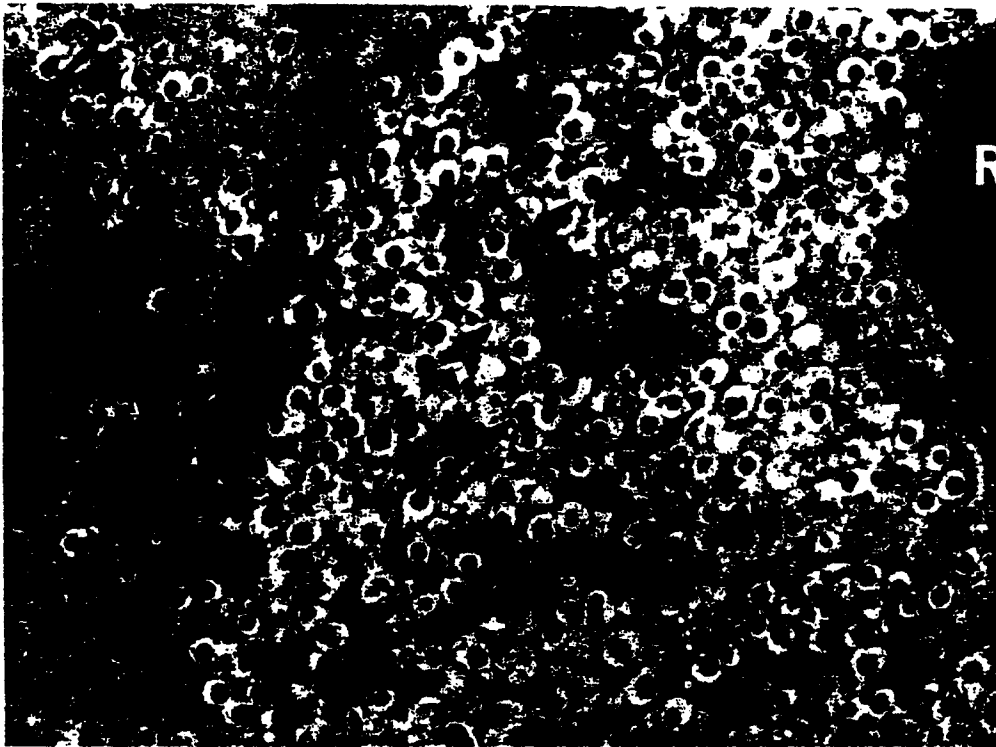


FIG. 8

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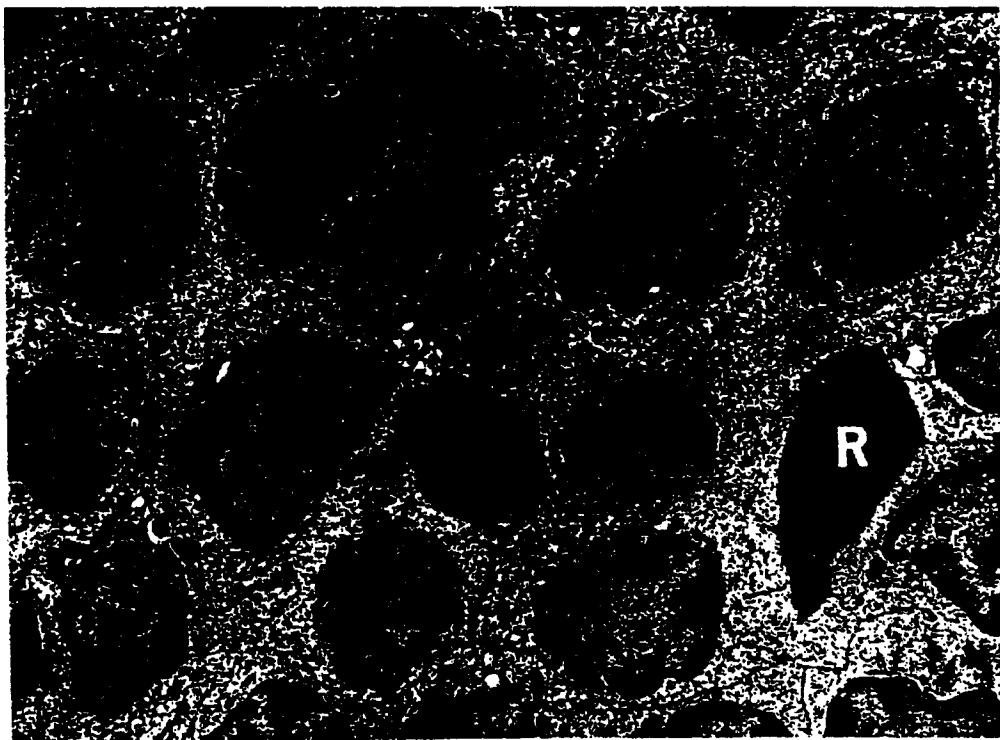


FIG. 9

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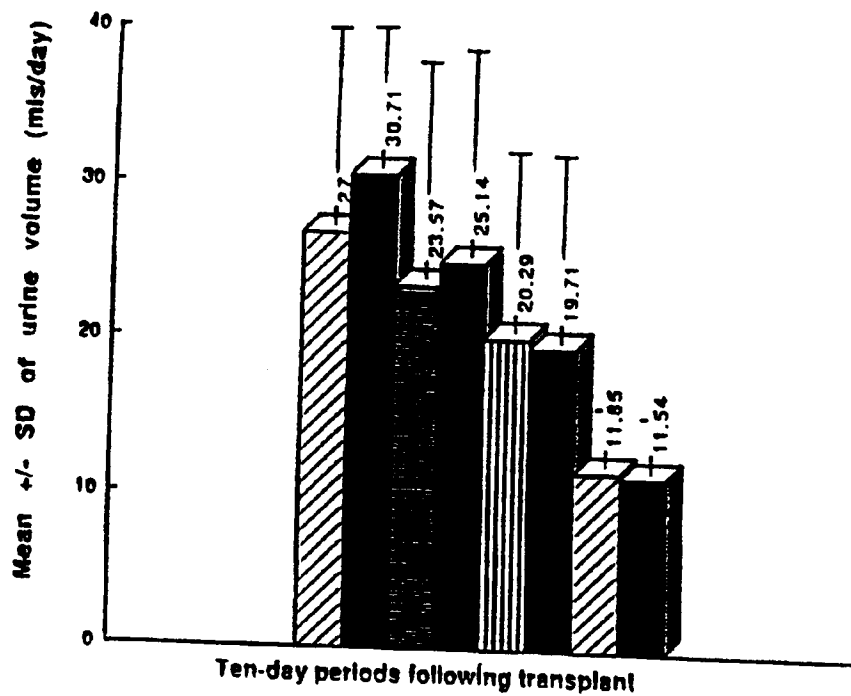


FIGURE 10

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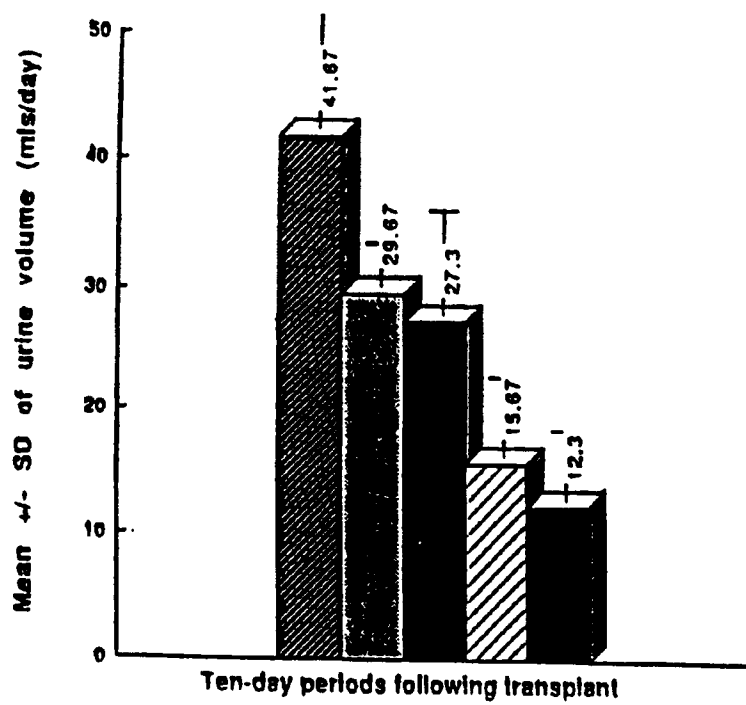


FIGURE 11

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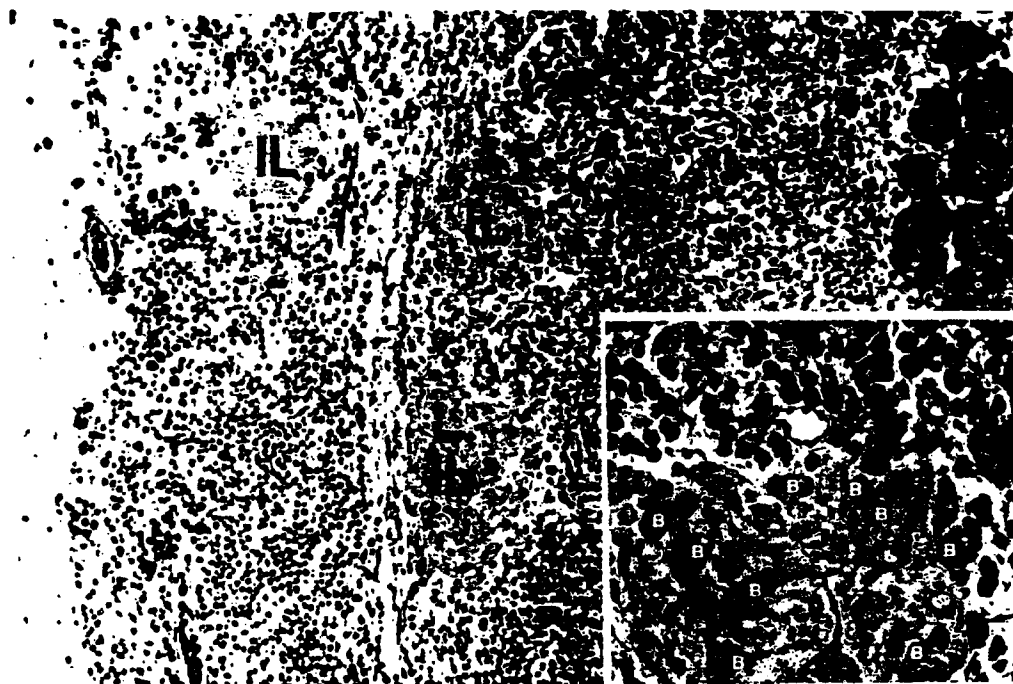


FIG. 12